NANOPARTICLES: POTENTIAL IN TREATMENT OF CANCER

ABSTRACT

THESIS

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BY

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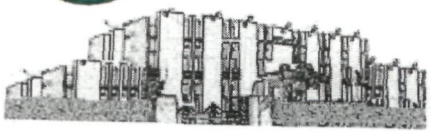

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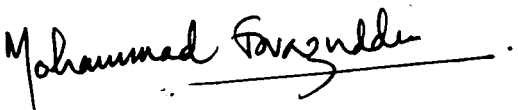
Certificate

I certify that research work presented in this thesis entitled **"Nanoparticles: potential in treatment of cancer"** has been carried out by Mr. Mohammad Farazuddin under my supervision, it is original in nature and is suitable for the award of Ph. D. degree in Biotechnology of the Aligarh Muslim University, Aligarh.

M. Owais, Ph. D.
(Associate Professor)

Declaration

I hereby declare that the thesis entitled **"Nanoparticles: potential in treatment of cancer"** embodies the research work carried out by me.



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Mohammad Farazuddin

(Mohammad Farazuddin)

CONTENT

Abstract

The occurrence of various forms of cancer has haunted mankind since prehistoric era. Modern lifestyle has only exaggerated the severity of the condition to mammoth levels. Despite continuous research on its early diagnosis, treatment to prevent or delay, this disease remains an unsolved puzzle. In a surge to discover new treatment modalities and therapeutics, various novel anti-cancer compounds and techniques have been developed. Though phytochemicals are considered to be wonder drugs of modern era having strong anti-cancer activities but are manifested with issues like solubility, stability and toxicity. Attainment of required concentration is another issue to be addressed. These associated problems retard their translation into clinical settings. Nanotechnology provides a solution to these dilemmas and to date many nano and micro sized systems such as nanoparticles, liposomes and micelles have been developed. Among these technologies, nanoparticles and liposomes have gained much insight. Variety of polymers have been synthesized with different degradation periods to fulfil the need. These polymers are biodegradable, biocompatible and do not induce any immunogenic response inside the host. Ongoing research has implemented drug delivery systems against cancer treatment, a series of strategies have been developed and modified to enhance the therapeutic efficacy of these nanoparticle and liposome based formulations. A number of obstacles in *in vivo* stability, bioavailability and non-targeted accumulation have impeded the clinical implication of these drug delivery systems. Those impediments have been partially resolved by scientific community significantly that resulted in various nanotechnology products in the market with enhanced efficacy. However, there are still certain demands to fulfil, particularly in the enhancement of tissue specificity and reduction in side effects. A principle barrier to destruction of disseminated cancer is its heterogeneous nature as most of the tumors

contain cell subpopulations that subvert the host defences and initiate angiogenesis on their own and bear self sufficiency in growth factors. So those are the few barriers that limit application of available chemotherapeutic agents that we need to overcome.

Most of the chemotherapeutic drugs fail to exhibit their desired effect as they do not reach at targeted site and are cleared from the systemic circulation before attainment of their required concentration. Therefore, high doses of the drugs need to be supplied that result in associated toxicity, another serious problem to tackle. There is consistent research on phytochemicals to provide an alternative to those available chemotherapeutic drugs. Plant derived compounds inhibit multiple signaling pathways and induce apoptosis. Few compounds specifically recognize and kill cancer cells sparing adjacent healthy cells. But these compounds possess palatability and solubility issues. Nanotechnology provides a platform to meet the challenges concerned with application of these therapeutics. One can use either active targeting or passive mode of delivery. A plethora of nanoparticles have been used to target and deliver the therapeutics in the tissues. Perillyl alcohol, a monoterpene possesses strong anti-cancer activity but has not been utilised for its efficacy.

In the first phase of the study, we encapsulated perillyl alcohol in poly lactic glycolic acid (PLGA) microparticles and characterized for their physical parameters like size, zeta-potential and release kinetics. Further, their toxicity was examined *in vitro* by RBC lysis test and *in vivo* by measuring renal and hepatic enzyme functions. Those formulations were evaluated for their inhibitory effect on epidermoid cancer cells (A253) *in vitro* and on di ethyl benzo anthracene (DMBA) induced skin papillomas. Characterization data revealed that PLGA microparticles were bigger in size and showed sustained release of POH. As confirmed by RBC lysis test, serum creatinine and alkaline phosphatase (ALP) estimation, POH bearing PLGA formulation bears toxicity either *in*

vitro or *in vivo*. Micro and nanoparticles are known to be taken up by cancer cells more avidly, thus releasing high drug payload. To confirm our hypothesis, we evaluated inhibitory effect of our developed formulation on epidermoid cancer cell (A253). POH bearing PLGA microparticles bear more cytotoxic effect on epidermoid cancer cells than free form of POH. Western blotting results of apoptotic factors (p21/Waf1 and Bax) revealed that POH bearing PLGA microparticles exerted more inhibitory effect *in vitro* whereas free form of the drug failed to induce similar inhibition. Further, those formulations were examined for their *in vivo* activity by observing reduction in papilloma size, mortality rate, apoptosis induction and skin architecture. POH bearing PLGA microparticles being more stable exhibited highest reduction in papilloma size (80%) followed by free form of drug (60%). They had upregulated expression of p53 wild type and p21/Waf1, resulting downregulation of p53 mutant expression. Survival data also indicated that POH bearing PLGA microparticles had maximum survival rate (80%) whereas free POH had only 40% survival rate. Skin tissue histology revealed that POH bearing PLGA microparticles regress skin papilloma much efficiently than free form of the drug. Sham PLGA had no effect on mortality, tumor regression and apoptosis induction and they behaved as untreated control animals. Our data clearly established that POH bearing PLGA microparticles successfully inhibited epidermoid cancer cells *in vitro* and induced tumor regression thus increasing the survival of treated animals.

Curcumin (di fruloyl methane) found in large quantities in the roots of *Curcuma longa*, has been used as a spice and colouring agent in Indian and Chinese food from centuries, also it has been utilised as a therapeutic agent. It possesses antioxidant, anti-inflammatory properties and inhibits chemically induced carcinogenesis in the skin, fore-stomach, colon, and liver. In spite of wonder molecule, its applications are limited because of hydrophobicity and poor absorption. In order to circumvent the

hydrophobicity hurdle of curcumin in its *in vivo* administration we developed a novel formulation. We encapsulated curcumin bearing PLGA microparticles in blank PC liposomes (microcells). Though PLGA microparticles also protect encapsulated molecules from external environment but they show burst release. We developed microcells and PLGA microparticulate formulation of curcumin, characterized them and evaluated their efficacy in treatment of di ethyl nitrosamine (DEN) induced hepatocellular carcinoma in model animals. Besides being safe, these formulations do not bear any toxicity constraint as revealed by *in vitro* and *in vivo* studies. Upon encapsulation of PLGA particles in blank PC liposomes, they were found to release curcumin in a much sustained manner than curcumin bearing PLGA microparticles, also they did not show any burst release. Curcumin bearing microcells and PLGA microparticles based formulations were found to distribute curcumin to the tissues comparatively better than the free curcumin. The potential of those formulations to treat hepatocellular carcinoma was assessed by evaluation of liver enzymes alkaline phosphatase (ALP), aspartate transaminase (AST) and gamma glutamyl transferase (GGT). Owing to their stability and sustained release pattern, curcumin bearing PLGA microcells, were found to reduce the liver enzymes much efficiently than PLGA microparticles. PLGA microparticles were superior in liver enzyme reduction over free curcumin. TNF-α, a known factor that mediates tumor cell differentiation and its survival was also examined in various curcumin formulations treated groups. Our results clearly established that microcells being more stable, released curcumin in a sustained manner and inhibited TNF-α expression more efficiently than PLGA microparticles. Free form of curcumin had very mild effect on TNF-α expression. We also elucidated the pathway involved in regression of HCC by curcumin bearing microcells and PLGA microparticles formulations. Treatment with curcumin bearing microcells and PLGA microparticles was found to

modulate p53 wild type, bax and bcl-2 expression. Most significant upregulation in p53 wild type and bax was observed in curcumin bearing microcells treated group formulations followed by PLGA microparticles. Also, those formulations were found to inhibit bcl-2 expression. Free form of curcumin did not modulate apoptotic factors much. Expression profile of p53 wild type, bax and bcl-2 established higher efficacy of curcumin bearing microcells and PLGA microparticles over free curcumin. Histological analysis also revealed that curcumin bearing microcells helped in regression of HCC and maintenance of cellular architecture of liver. Curcumin bearing PLGA microparticles were also found to regress liver cancer to some extent but not as significant as microcells. Sham microcells and PLGA microparticles had no effect neither on modulation of apoptotic factors nor on maintenance of tissues architecture. Ergo, we can conclude that encapsulation of curcumin in microcells and PLGA microparticles, increases its solubility and bioavailability. These formulations were found to inhibit HCC growth efficiently in Swiss albino mice.

In the last chapter of my thesis I have tried to overcome siRNA stability issue for its possible delivery inside cancer cells. With increasing knowledge of molecular mechanism of gene silencing using RNA interference, small interfering RNA (siRNA) is now used for the treatment of incurable diseases like cancer. There are certain challenges to be accomplished before translation of siRNA therapy in clinical settings. Unspecific targeting, premature degradation and targeted delivery are few major challenges to resolve. Delivery of therapeutics at required site plays an important role as most of treatment modalities require systemic administration. siRNA delivery using nanotechnology based tools could be one such plausible solution for safe and target specific delivery of siRNA. COX-2 (Cyclo Oxygenase 2) is an important enzyme that helps in prostaglandin synthesis and has been reported to mediate carcinogenesis and

angiogenesis in various cancer types. We developed minicells from *Bacillus subtilis* total lipid for the delivery of COX-2 siRNA and compared their efficacy with phosphatidyl choline (PC) liposomes. They were used to treat DEN induced hepatocarcinogenesis in model animals. Liposome mediated delivery of COX-2 siRNA helped in liver enzyme reduction *viz* aspartate transaminase (AST) and alanine transaminase (ALT), and tumor regression in treated animals. Apoptosis measurement via APO-Brdu staining of liver cells also indicated that minicells bearing COX-2 inhibited DEN induced carcinogenesis much efficiently than PC liposomes. In addition, Western blotting of COX-2 and apoptotic molecules (p53 wild type and bax) revealed that minicells encapsulated COX-2 siRNA downregulated the expression of COX-2 significantly than PC liposomes and free siRNA. Upregulation of p53 wild type and bax and downregulation of bcl-2 expression was as a result of COX-2 inhibition. Histology of liver tissues and survival data also established that minicells mediated delivery of COX-2 siRNA helps in tumor regression, cellular morphology maintenance, and increases in survival of treated animals. Though free siRNA also imparts protection, increases survival and initiates apoptosis but not as significantly as minicells encapsulated siRNA and PC liposomes did. Minicells (*B.subtilis* liposomes) being more stable exhibited comparatively higher regression and increased survival than PC liposomes. Our results demonstrated that minicells and PC liposomes mediated delivery of COX-2 siRNA saves it from premature degradation and successfully inhibits hepatocarcinogenesis in model animals.

We can conclude that nanotechnology offers very promising tools that can be used for passive targeting. Various nanoparticle and liposome based approaches can be applied to overcome the solubility and bioavailability issues of drugs. Also those approaches can be availed for tissue specific delivery of siRNA molecules.

Abbreviations

GCT	Germ cell tumor
HPV	Human papilloma virus
EBV	Epstein barr virus
HHV	Human herpes virus
CFC	Chloro fluoro carbon
VEGF	Vascular endothelial growth factor
FGF	Fibroblast growth factor
POH	Perillyl alcohol
PNA	Peptide nucleic acid
BCA	Bio barcode amplification
QD	Quantum dots
CNT	Carbon nanotubes
AFM	Atomic force microscopy
MPS	Mononuclear phagocytic system
EPR	Enhanced permeability and retention effect
RES	Reticular endothelial system
MMP	Matrix metallo proteinase
DOX	Doxorubicin
MDR	Multiple drug resistance
DACH	Di-amino cyclo hexane
HPMA	n-(2-hydroxy propyl) methyl acrylamide
PGA	Poly glutamic acid
AMD	Age related macular degeneration
RSV	Respiratory syncytial virus
shRNA	Short hairpin RNA
dsRNA	Double stranded RNA
TRBP	TAR RNA binding protein
AGO-2	Argonaute 2
RISC	RNA inducing silencing complex
TLR	Toll like receptor
DNP	Di-nitro phenol
CTL	Cytotoxic T-Lymphocytes
PLGA	Poly-lactic glycolic acid

HSA	Human serum albumin
DMBA	Di-methyl benzo anthracene
PVA	Poly vinyl alcohol
DCM	Di-chloro methane
W/O/W	water-in-oil-in-water
SEM	Scanning electron microscopy
LSD	Least Statistical Difference
HCC	Hepatocellular carcinoma
DEN	Di-ethyl nitrosamine
ALP	Alkaline phosphatase
AST	Alanine transferase
GGT	Gamma glutamyl transferase
PC	Phosphatidyl choline
TNF	Tumor necrosis factor
PBS	Phosphate buffer saline
NS	Normal saline
COX-2	Cyclo oxygenase 2

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Chapter I: Review of literature

(Part I: Cancer)

1.1 Cancer: A complex worldwide scourge

Cancer, or malignant neoplasm in medical definition, is the name given to a class of complex diseases in which groups of bodily cells display uncontrolled growth that eventually leads to invasion of adjacent tissues, and sometimes metastasis, spreading to other locations in the body via lymph or blood. The spread of the malignant cells extends outward from the original tumor, and has been described as resembling the appearance of a crab. This is the origin of the term 'cancer', which was derived from the Latin meaning 'crab' (**Walter 1977**). Cancer is caused by genetic instability and accumulation of multiple molecular alterations (**Hanahan & Weinberg 2000, Hahn & Weinberg 2002**). Any cell has the potential to undergo malignant changes and develop into cancer. This can occur when 'normal' cell control mechanisms become disrupted or indeed fail (**Figure 1.1**) (**Corner 2001**). The complexity of cancer is compounded by the current lack of effective diagnostic and prognostic tools that are crucial for the prediction of clinical heterogeneity of tumors and are insufficient for successful treatment and patient outcome (**Liotta & Petricoin 2000, Petricoin 2002**).

Because of the lack of diagnosis, at the time of clinical presentation, more than 60% of patients with cancer have hidden or overt metastatic colonies (**Menon & Jacobs 2000**). At this stage, therapeutic modalities are limited in their effectiveness. Due to these problems, cancer has overtaken heart disease as the leading cause of death for adults in the United States (**Figure 1.2**) [**United States Cancer Statistics, Centers for Disease Control and Prevention (CDC)** http://www.cdc.gov/cancer/npcr/uscs]. The occurrence of cancer cases has been recorded since prehistoric era; modern life style has only exaggerated the condition. In 2008 approximately 12.7 million cancers were diagnosed and 7.6 million people died of cancer worldwide (**Jemal *et al.* 2011**). Cancers as a group account for approximately 13% of all deaths each year with the most common being lung, stomach, colorectal, liver, and breast cancer (**WHO 2006**). This makes invasive cancer the leading cause of death in the developed world and the second leading cause of death in the developing world (**Jemal *et al.* 2011**).

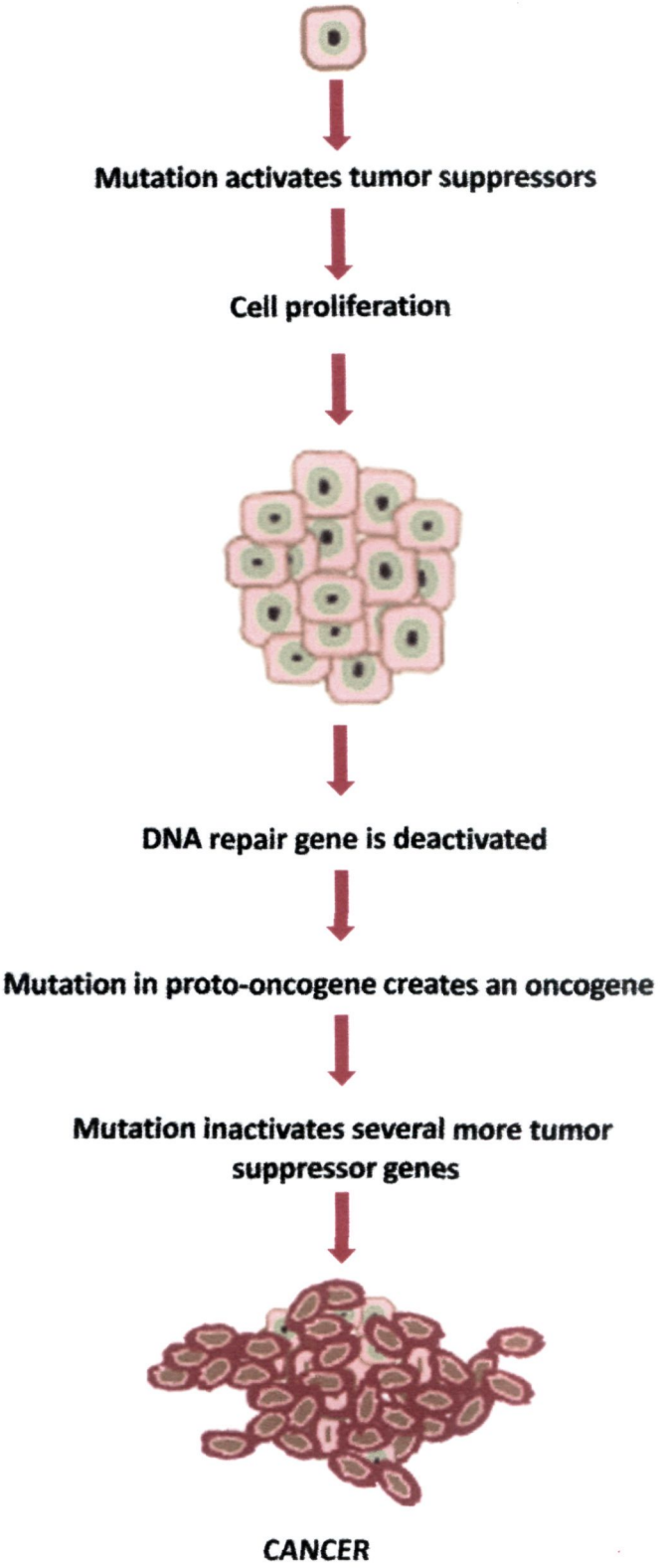


Figure 1.1 Mutation in tumor suppressor gene leads to uncontrolled growth and cancer formation.

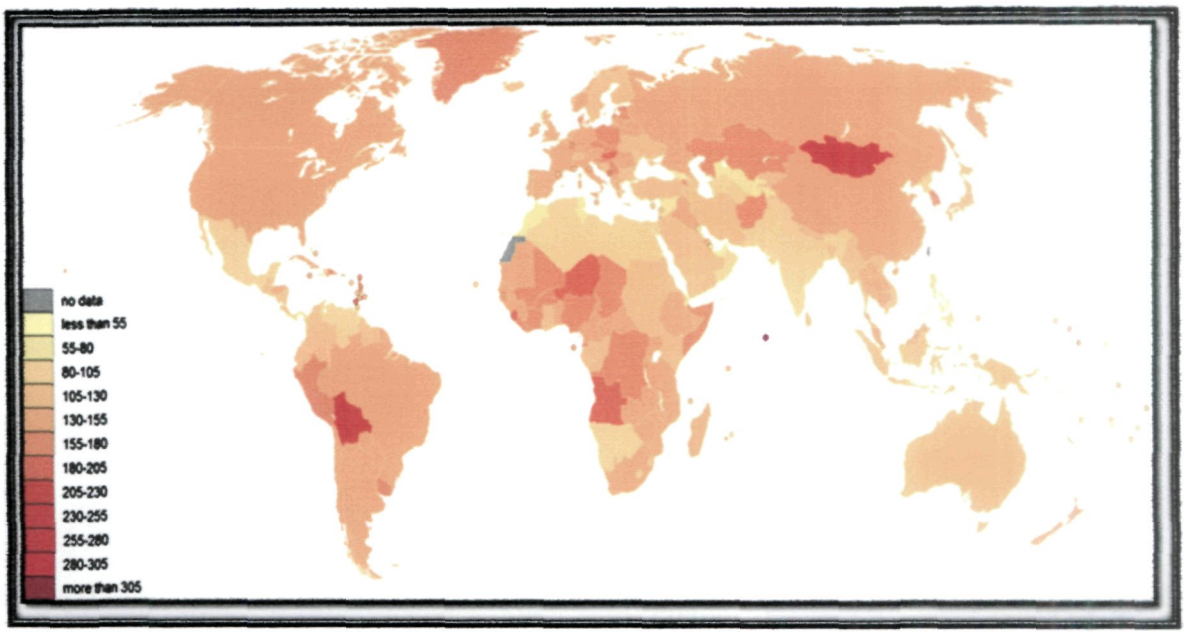


Figure 1.2 Cancer epidemiology worldwide. (Death rate from malignant cancer per 100,000 inhabitants in 2004) **(WHO 2009).**

1.2 Classification

I. **Lymphoma** is cancer of lymphatic cells of the immune system. Typically, lymphomas are present as a solid tumor of lymphoid cells. These malignant cells often originate in lymph nodes, presenting as an enlargement of the node (a tumor). It can also affect other organs in which case it is referred to as extra nodal lymphoma. Extra nodal sites include the skin, brain, bowels and bone. Lymphomas are closely related to lymphoid leukemia, which also originate in lymphocytes but typically involve only circulating blood and the bone marrow (where blood cells are generated in a process termed haematopoesis) and do not usually form static tumors.

II. Cancer of blood or bone marrow, characterized by abnormal increase of white blood cells is called as **Leukemia.** Leukemia is a broad term covering a spectrum of diseases. In turn, it is part of the even broader group of diseases called hematological neoplasm.

III. **Germ cell tumor** (GCT) is a neoplasm derived from germ cells. Germ cell tumors can be cancerous or non-cancerous. Germ cells normally occur inside the gonads (ovary and testis). Germ cell tumors that originate outside the gonads may be birth defects resulting from errors during development of the embryo.

IV. **Blastoma** is caused by malignancies in precursor cells, often called blasts. Examples are nephroblastoma, medulloblastoma and retinoblastoma. The suffix blastoma is used to imply a tumor of primitive, incompletely differentiated (or precursor) a cell, *i.e,* chondroblastoma is composed of cells resembling the precursor of chondrocytes.

1.3 Causes of Cancer

Cancer may be called an environmental disease as 90-95% of cases are due to environmental factors and merely 5-10% due to genetics (**Anand** *et al.* 2008). "Environmental factors", include everything from natural sunlight to industrial pollution to viruses to behavioral choices to old age. Most environmental causes, such as naturally occurring background radiation, are not modifiable or controllable. Common environmental factors that lead to cancer death include: tobacco (25-30% of deaths), diet and obesity (30-35%), infections (15-20%), radiation (both ionizing and non ionizing), stress and lack of physical activity (**Anand** *et al.* 2008).

I. Diet

It is well recognized that a diet that is low in fiber and high in animal fats can significantly contribute to the development of colorectal cancer (**Cartmel & Reid 2000, Yarbro & Frogge *et al.* 2005**). It is estimated that a third of all cancers and possibly as many as 90% of colorectal malignancies are directly associated to diet (**Yarbro *et al.* 2005**). The development of cancer through dietary factors is not confined to colorectal malignancies. Diet which is high in fat content leads to the development of breast and stomach cancer also (**Willett 1989, Sheppard 2001**).

Tobacco: Decades of research has demonstrated the relation between tobacco use and cancer in the lung, larynx, head, neck, stomach, bladder, kidney, oesophagus and pancreas (**Kuper *et al.* 2002**). Tobacco smoke contains over fifty known carcinogens, including nitrosamines and polycyclic aromatic hydrocarbons (**Kuper *et al.* 2002**). There is overwhelming evidence to link tobacco with a variety of cancers. Tobacco smoking is associated with many forms of cancer (**Sasco *et al.* 2004**), and causes 90% of lung cancer (**Biesalski *et al.* 1998**). The malignant conditions linked to the use of/exposure to tobacco includes (**Horton-Taylor 2001**):

- Small cell (oat cell) carcinoma of the lung
- Oropharnygeal cancer
- Bladder cancer
- Cervical cancer
- Gastric cancer
- Lip cancer
- Pancreatic cancer

II. Infection

Some cancers can be caused by infection. Upto 20% of total human cancers are caused by infections (**Pagano *et al.* 2004**). Viruses are the most common infectious agents that cause cancer. Bacterial infection may also increase the risk of cancer, as seen in *Helicobacter pylori* induced gastric carcinoma (**Pagano *et al.* 2004**). Parasitic infections strongly associated with cancer are caused mainly by *Schistosoma haematobium* (squamous

cell carcinoma of the bladder) and the liver flukes, *Opisthorchis viverrini* and *Clonorchis sinensis* (cholangiocarcinoma) (**Samaras *et al.* 2010**).

A. Viruses

Viruses are one of the most important risk factor second to tobacco for cancer development in humans (**zur Hausen 1991**).

There are two types of cancers caused by viruses:

a) **Acutely transforming viruses:** the virus carries an overactive oncogene, and the infected cell becomes cancerous as soon as the overactive viral gene is expressed.
b) **Slowly transforming viruses:** the virus genome is inserted near a previously existing proto-oncogene in the genome of the infected cell. The virus causes overexpression of that proto-oncogene, which typically induces uncontrolled cell division. Because the virus genes might not insert near enough to a proto-oncogene to trigger the cancerous changes, and, even if optimally located, it might take some time to become activated; slowly transforming viruses usually cause tumors much longer after infection than the acutely transforming viruses.

People with chronic hepatitis B infection are more than 200 times more likely to develop liver cancer than uninfected people (**Sung *et al.* 2000**). Liver cancer is one of the most common causes of cancer-related deaths in the world, and is especially common in East Asia and parts of sub-Saharan Africa.

Human papilloma viruses (HPV) are another particularly common cancer-causing virus. HPV is well-known for causing genital warts and essentially all cases of cervical cancer, but it can also infect and cause cancer in several other parts of the body, including the larynx, lining of the mouth, nose, throat, anus, and esophagus. **Herpes viruses** are a third group of common cancer-causing viruses. Two types of herpes viruses have been associated with cancer: the **Epstein-Barr virus** (EBV) and **human herpes virus 8** (HHV-8) (**Cohen & Jeffrey I 2000**). EBV appears to cause all non keratinizing nasopharyngeal carcinomas and some cases of lymphoma, including Burkitt's lymphoma (**Cohen & Jeffrey I 2000**). EBV has also been found in a variety of other

types of cancer cells, although its role in causing these other cancers is not well established. HHV-8 causes all cases of Kaposi's sarcoma.

HIV does not directly cause cancer, but it is associated with a number of malignancies, especially Kaposi's sarcoma, non-Hodgkin's lymphoma, anal cancer and cervical cancer. Kaposi's sarcoma is caused by human herpes virus 8. AIDS-related cases of anal cancer and cervical cancer are commonly caused by human papilloma virus. After HIV destroys the immune system, the body is no longer able to control these viruses, and the infections manifest as cancer (**Wood & Harrington 2005**).

B. Bacteria

Helicobacter pylori can live in the lining of the stomach and is linked to the development of gastric and duodenal ulcers. It is now known that there is a link between chronic ulcer disease and the development of malignancy.

C. Parasites

The parasites that cause schistosomiasis (bilharzia), especially *S. haematobium*, can cause bladder cancer and cancer at other sites (**Mustacchi 2000**). Inflammation triggered by the worm's eggs appears to be the mechanism by which squamous cell carcinoma of the bladder is caused (**Mustacchi 2000**).

III. Radiation

Sources of ionizing radiation, such as radon gas, can also lead to cancer. Radiation can cause cancer in most parts of the body, in all animals, and at any age, although radiation-induced solid tumors usually take 10-15 years, and up to 40 years, to become clinically manifest, and radiation-induced leukemias typically require 2-10 years to appear (**Little & John B 2000**). Low-dose exposures, such as living near a nuclear power plant, are generally believed to have no or very little effect on cancer development (**Little & John B 2000**). Radiation is a more potent source of cancer when it is combined with other cancer-causing agents, such as radon gas exposure plus smoking tobacco (**Little & John B 2000**). Major damage normally results in the cell dying, but smaller damage may leave a stable, partly functional cell that may be capable of proliferating and developing into

cancer, especially if tumor suppressor genes were damaged by the radiation **(Little & John B 2000)**.

Prolonged exposure to ultraviolet radiation from the sun can lead to melanoma and other skin malignancies. Clear evidence establishes ultraviolet radiation, especially the medium wave UVB, as the cause of most non-melanoma skin cancers, which are the most common forms of cancer in the world **(Cleaver *et al.* 2000)**.

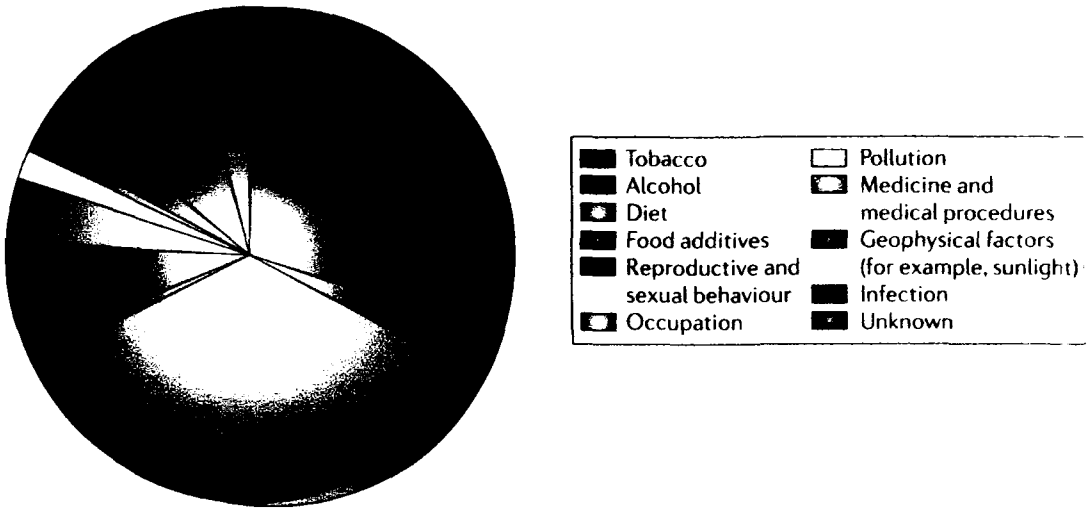


Figure 1.3 Proportion of cancer deaths attributed to non-genetic factors. Estimated proportion of cancer in the United States in category of non-genetic cancer causes **(Adapted from Doll and Peto, 1981)**.

Exposure to ionizing radiation can result in the following malignancies:

- leukemia
- thyroid cancer
- squamous cell carcinoma of the skin.

IV. Pollution

Increasing use of chlorofluorocarbons (CFCs) is leading to the destruction of the ozone layer, resulting in more ultraviolet radiation reaching the earth. This is a contributing factor to the increase in the number of skin cancers. Also uncompleted combustion of

coal and certain other materials generate di-methyl benzo anthracene and other carcinogens which causes skin and other type of malignancies.

V. Chemicals

In the United States, extensive epidemiological data had documented about the carcinogenic hazards of workplace exposures, including asbestos, benzene, arsenic, nickel, polycyclic hydrocarbons and vinyl chloride (**Colditz *et al.* 2006**).

Millions of workers run the risk of developing cancers such as lung cancer and mesothelioma from inhaling asbestos fibers and tobacco smoke, or leukemia from exposure to benzene at their workplaces (**WHO, 2007**). The onset of the resulting malignancy, that is mesothelioma, could run over a period of many years from the initial exposure. The use of asbestos has now been greatly reduced.

VI. Genetics

With the development of molecular biology, new techniques are leading to the identification of genes which increase an individual's predisposition to develop cancer. Genes already identified include (**Yarbro *et al.* 2005**):

- RBI Retinoblastoma
- WT1 Wilm's tumor
- APC Familial polyposis
- CDKN2 Dysplastic nevus syndrome
- FACC Fanconi's anemia
- BLM Bloom syndrome (associated with leukemia)
- BRCA1 Breast and ovarian cancer
- BRCA2 Breast cancer

Table 1.1: Chemicals/agents linked to specific cancers

Chemical/Agent	Cancer
Asbestos	Mesothelioma
Pitch, soot, coal tar, oil	Squamous cell carcinoma of the skin, scrotum
Vinyl chloride	Liver
Arsenic Sinuses	lung
Benzidine	Bladder
Wool/leather/wood dust	Nasal sinuses
Aniline dyes	Bladder

1.4 Molecular mechanism of cancer invasion

Cancer cell lack regulation in its machinery that operates its division and proliferation. They divide and proliferate infinitely and bypass the apoptosis. Following are six basic alterations in a normal cell's physiology that lead to tumor development.

1. Acquired capability: self sufficiency in growth signal

A healthy cell requires growth signals to move from one stage to another. Those signals are translated via cell bound transmembrane receptors that bind with distinct class of signaling molecules: diffuse growth factors, extracellular matrix components, and cell to cell adhesion molecules. Cancer cells have the potential to mimic those growth signals and possess self sufficiency in proliferation.

2. Insensitivity to growth signals

Within a normal tissue, multiple anti-proliferative signals operate to maintain cellular quiescence and tissue homeostasis; these signals include both soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix and on the surfaces of nearby cells. These growth-inhibitory signals, like their positively acting counterparts, are received by transmembrane cell surface receptors coupled to intracellular signaling circuits. Cancer cells bypass this mechanism by inhibiting those growth signals.

3. Sustained angiogenesis

Angiogenesis is the physiological process involving the growth of new blood vessels from pre-existing vessels. Tumors induce blood vessel growth (angiogenesis) by secreting various growth factors (VEGF). Growth factors such as bFGF and VEGF can induce capillary growth into the tumor.

4. Evading apoptosis

The tumor-suppressor protein p53 accumulates when DNA is damaged due to a chain of biochemical factors. Part of this pathway includes α-interferon and β-interferon, which induce transcription of the p53 gene and result in the increase of

p53 protein level and enhancement of cancer cell-apoptosis (**Takaoka *et al.* 2003**). p53 prevents the cell from replicating by stopping the cell cycle at G1, or interphase, to give the cell time to repair, however it will induce apoptosis if damage is extensive and repair efforts fail. Any disruption in the regulation of the *p53* or interferon gene, result in impaired apoptosis and the possible formation of tumors.

5. Limitless replicative potential

Three acquired capabilities: growth signal autonomy, insensitivity to antigrowth signals, and resistance to apoptosis-all lead to an uncoupling of a cell's growth program from signals in its environment. Most types of tumor cells that are propagated in culture appear to be immortalized, suggesting that limitless replicative potential is a phenotype that was acquired *in vivo* during tumor progression and was essential for the development of their malignant growth state (**Hayflick 1997**). This result suggests that at some point during the course of multistep tumor progression, evolving premalignant cell populations exhaust their endowment of allowed doublings and can only complete their tumorigenic agenda by breaching the mortality barrier and acquiring unlimited replicative potential.

6. Tissue invasion and metastasis

Once tumor is formed, it starts invading the neighbor tissues and cancerous cell migrate through newly formed blood vessels and spreads in other organs of the body. This distant settlement of tumor cells is called as metastasis.

1.5 Metastatic spread

Surgical removal of the original tumor is not always a successful treatment in malignant disease, due to microscopic spread. Malignant tumors are often irregular in shape, with ill-defined margins (**Wolfe 1986, Walter 1977**). The potential for microscopic spread occurs when the tissue surrounding the visible tumor appears to the eye (macroscopic examination) to be unaffected by cancer. Microscopic examination of the surgical resection margins can reveal the presence of malignant cells. If left untreated, these cells will result in localized recurrence of the cancer and eventual spread (metastasis).

As malignant cells replicate, they grow in an irregular pattern, infiltrating surrounding tissue which results in infiltration of the lymphatics and/or blood vessels. By gaining access to these vessels, malignant cells can be carried to other sites within the patient's body, where they replicate and grow (**Figure 1.4**) (**Wolfe 1986, Walter 1977**). In order to ensure that these malignant cells receive the nourishment they need to thrive, angiogenesis occurs. This is the formation of new blood vessels (**Yarbro** *et al.* 2005).

I. Lymphatic Spread

Malignant cells gain access to the lymphatic system and travel along the vessels to the 'regional draining' lymph nodes (**Walter 1977**). The malignant cells establish themselves in regional nodes, where they replicate and eventually replace the lymph node with a malignant tumor that is, cancer. Malignant cells from this tumor can then travel, via the lymphatic system, to the next group of lymph nodes, thereby spreading the malignancy throughout the body (**Walter 1977**). Lymphomas and squamous cell carcinoma of the head and neck are two examples of where cancer commonly spreads via the lymphatic system (**Yarbro** *et al.* 2005).

II. Blood Spread

Malignant cells can also infiltrate the vascular system and travel along the vessels until they arrive at an area where they can become lodged and subsequently replicate to form a secondary (metastatic) deposit. The malignant cells can then migrate via the smaller blood vessels that is, the capillaries (**Walter 1977**). However, there is evidence that only a small percentage of cells entering the vascular system actually survive to give rise to blood-borne metastatic spread (**Walter 1977**). Malignancies which are linked to blood-borne spread include melanoma and small cell carcinoma of the lung (**Yarbro** *et al.* 2005).

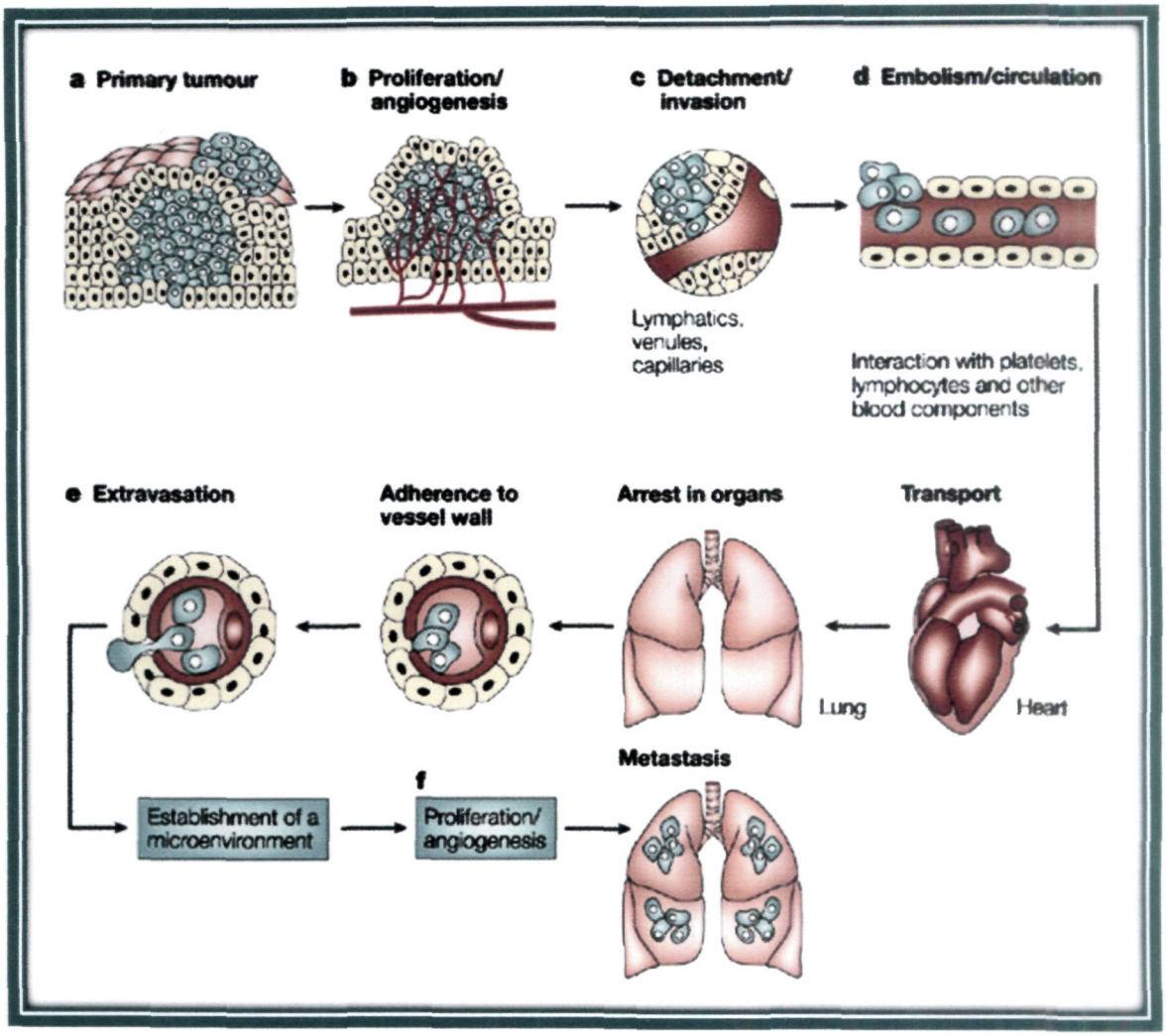


Figure 1.4 Metastatic spread: Primary tumor formation leads to angiogenesis, which eventually results in detachment and circulation of cancerous cells to other part of body.

1.6 Treatment strategies for cancer

Deep knowledge of cancer invasion and its proliferation has given rise to various technologies for the treatment of cancer. The choice of therapy depends upon the location and grade of the tumor and stage of the disease. Complete removal of the cancer without damage to the rest of the body is the goal of treatment. Sometimes this can be accomplished by surgery, but the propensity of cancers to invade adjacent tissue or to spread to distant sites by microscopic metastasis often limits its effectiveness. The effectiveness of chemotherapy is often limited by toxicity to other tissues in the body. Radiation can also cause damage to normal tissue. Following are the few treatment modalities used for the treatment.

A. Radiation therapy

Radiation therapy uses high energy radiation to kill cancer cells by damaging their DNA (**Lawrence *et al.* 2008**). X-rays, gamma rays, and charged particles are types of radiation used for cancer treatment. The radiation used for cancer treatment may come from a machine outside the body, or it may come from radioactive material placed in the body near tumor cells or injected into the bloodstream. This is called as **internal radiation therapy**. **Systemic radiation therapy** uses radioactive substances, such as radioactive iodine, that travel in the blood to kill cancer cells. Some patients receive radiation therapy alone, and some receive radiation therapy in combination with chemotherapy. The main side effect of radiation therapy is that it not only kills cancerous cells but also healthy cells that lie adjacent to them. Also if few cells are left irradiated, they tend to expand with time. So while performing radiation therapy it must be planned carefully and executed.

B. Surgery

Non-hematological cancers can be cured with the help of surgery, but this is only possible when tumor has not metastasized. Tumors grow locally, and then spread to the lymph nodes, then to the rest of the body. Even small localized tumors are increasingly recognized as possessing metastatic potential.

Cryosurgery is a technique for freezing and killing abnormal cells. It is used to treat some kinds of cancer and some precancerous or noncancerous conditions, and can be used both inside the body and on the skin. Cryosurgery is an alternative to surgery for liver cancer that has not spread, for cancer that has spread to the liver from another site, for prostate cancer confined to the prostate gland, for a precancerous condition of the cervix, and for cancerous and noncancerous tumors of the bone. Cryosurgery may have fewer side effects than other types of treatments, and is less expensive and requires shorter recovery times. The technique is still under study, and its long-term effectiveness is not known.

C. Targeted therapies

Targeted therapy, which first became available in the late 1990s, has had a significant impact in the treatment of some types of cancer, and is currently a very active research area. This constitutes the use of agents specific for the deregulated proteins of cancer cells. Small molecule targeted therapy drugs are generally inhibitos of enzymatic domains on mutated, over-expressed, or otherwise critical proteins within the cancer cell. Prominent examples are the tyrosine kinase inhibitors **imatinib** (Gleevec/Glivec) and **gefitinib** (Iressa). Monoclonal antibody therapy is another strategy in which the therapeutic agent is an antibody which specifically binds to a protein on the surface the cancer cells. Examples include the **anti-HER2/neu antibody trastuzumab** (Herceptin) used in breast cancer, and the **anti-CD20 antibody rituximab**, used in a variety of B-cell malignancies. Targeted therapy can also be done using small peptides **(Zennami *et al.* 2011)**, and aptamers **(Ogris & Wagner 2011)** that specifically target over expressed cell surface receptors or affected extracellular matrix surrounding the tumor. Radionuclides which are attached to these peptides (e.g. RGDs) eventually kill the cancer cell if the nuclide decays in the vicinity of the cell. Especially oligo- or multimers of these binding motifs are of great interest, since this can lead to enhanced tumor specificity and avidity.

D. Immunotherapy

Immunotherapy is a strategy that is designed to induce immune system of host to fight against the disease. Contemporary methods for generating an immune response against tumors include intravesical BCG immunotherapy (**Didilescu 2010**), use of interferons (**Hervas-Stubbs *et al.* 2011**) and other cytokines (**Nicholas & Lesinski 2011**) to induce an immune response. Vaccines to generate specific immune responses are the subject of intensive research for a number of tumors, notably malignant melanoma and renal cell carcinoma. Sipuleucel-T is a vaccine-like strategy in late clinical trials for prostate cancer in which dendritic cells from the patient are loaded with prostatic acid phosphatase peptides to induce a specific immune response against prostate-derived cells.

E. Hormonal therapy

The growth of some cancers can be inhibited by providing or blocking certain hormones. Common examples of hormone sensitive tumors include certain types of breast and prostate cancers. Removing or blocking estrogen or testosterone is often an important additional treatment. In certain cancers, administration of hormone agonists, such as progestogens may be therapeutically beneficial.

F. Angiogenesis inhibitors

Angiogenesis inhibitors prevent the extensive growth of blood vessels (angiogenesis) that tumors require to survive. Few drugs (**Thapa *et al.* 2011**) and monoclonal antibodies (**Damasceno 2011**) have been used to prevent angiogenesis in cancer tissues. Some, such as bevacizumab, have been approved and are in clinical use. But one of the main problems with anti-angiogenesis therapy is that many factors stimulate blood vessel growth in cells normal or cancerous. Anti-angiogenesis therapy only targets one factor, so the other factors continue to stimulate blood vessel growth. Other problems include route of administration, maintenance of stability, activity and targeting at the tumor vasculature (**Kleinman & Liau 2001**).

G. Chemotherapy

Chemotherapy is the treatment of cancer with anticancer drugs that can destroy cancer cells. Chemotherapeutic drugs interfere with cell division in various possible ways, e.g. with the duplication of DNA or the separation of newly formed chromosomes. Most forms of chemotherapy target all rapidly dividing cells and are not specific to cancer cells, although some degree of specificity may come from the inability of many cancer cells to repair DNA damage, while normal cells generally can. Hence, chemotherapy has the potential to harm healthy tissue, especially those tissues that have a high replacement rate (e.g. intestinal lining). These cells usually repair themselves after chemotherapy. Sometimes drugs are given in combination as they show better effect than alone, called as "**combination chemotherapy**".

1.7 Phytochemicals in chemotherapy

Cancer chemotherapeutic agents can often provide temporary relief from symptoms, prolongation of life and occasionally, cures. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells. This ideal situation is achievable by inducing apoptosis in cancer cells. The life span of both normal and cancer cells is significantly affected by the rate of apoptosis. Thus, modulating apoptosis may be useful in the management and therapy of cancer. Synthesis or modification of known drugs continues as an important aspect of research. However, a vast amount of synthetic work has contributed relatively small improvements over the prototype drugs. There is a continued need for new prototypes to use in the design of potential chemotherapeutic agents. Significantly, natural products are providing such templates.

Recent studies on tumor inhibitory compounds of plant origin have yielded an impressive array of novel structures. Besides, epidemiological studies suggest that consumption of diets containing fruits and vegetables, major sources of phytochemicals and micronutrients, may reduce the risk of developing cancer. Certain products from plants are known to induce apoptosis in neoplastic cells but not in normal cells (**Hirano *et al.* 1995, Jiang *et al.* 1996**). Those phytochemicals modulate different processes and shows cumulative inhibitory effect. Also they bear less or no toxicity constraint.

I. Alkylating agents

Alkylating agents function by forming covalent bonds with the amino, carboxyl, sulfhydryl, and phosphate groups in biologically important molecules. Cisplatin and carboplatin as well as oxaliplatin are alkylating agents. Other agents include mechlorethamine, cyclophosphamide, clorambusil and infosfamide.

II. Anti-metabolites

Anti-metabolites masqueradeas purines (azathioprine, mercaptopurine) or pyrimidines, which become the building blocks of DNA. They prevent these substances from becoming incorporated in to DNA during the "S" phase (of the cell cycle), stopping normal development and division. They also affect RNA synthesis. Due to their efficiency, these drugs are the most widely used cytostatics.

III. Plant alkaloids

Alkaloids are derived from plants and block cell division by preventing microtubule function. Microtubules are vital for cell division and its inhibition blocks the process. Examples are vinca alkaloids and taxanes. They are derived from the Madagascar periwinkle, *Catharanthus roseus* (formerly known as *Vinca rosea*). The vinca alkaloids include: Vincristine, Vinblastine, Vinorelbine and Vindesine.

IV. Terpenoids

Terpenoids are another category of phytochemicals that possess strong anti-cancer activity. Monoterpenes such as D-limonene and perillyl alcohol (POH) derived from orange peels and lavender, respectively; possess chemopreventive properties against mammary, liver and lung carcinogenesis.

V. Podophyllotoxin

Podophyllotoxin is a plant derived compound which is said to help with digestion as well as used to produce two other cytostatic drugs, etoposide and teniposide.

They prevent the cell from entering the G1 phase (the start of DNA replication) and the replication of DNA (the S phase). The exact mechanism of its action is not yet known.

VI. **Taxanes**

The prototype taxane is the natural product paclitaxel, originally known as Taxol and first derived from the bark of the Pacific Yew tree. Docetaxel is a semi-synthetic analogue of paclitaxel. Taxanes enhance stability of microtubules, preventing the separation of chromosomes during anaphase.

VII. **Topoisomerase inhibitors**

Topoisomerases are essential enzymes that maintain the topology of DNA. Inhibition of type I or type II topoisomerases interferes with both transcription and replication of DNA by upsetting proper DNA supercoiling.

A. Type I topoisomerase inhibitors include campothecins: irinotecan and topotecan.

B. Type II inhibitors include amsacrine, etoposide, etoposide phosphate and teniposide

VIII. Polyphenols

The human diet contains a complex mixture of plant polyphenols. Studies have shown that cytotoxic effect of these phenols against different tumors is mediated through apoptosis (**Inoue *et al.* 1994**). Gallic acid, curcumin and caffeic acid are known polyphenols that inhibit cancer cells proliferation (**Reuter *et al.* 2011**).

IX. Flavonoids

Flavonoids varied significantly in their antiproliferative potency depending on the structural features. Kuntz et al. found that flavonoids baicalein and myricetin were able to induce apoptosis in HT-29 and Caco-2 cells. Flavonoids of the flavone, flavonol, flavanone and isoflavone classes possess antiproliferative effects in different cancer cell lines (**Kuntz *et al.* 1999**).

Part II:
Nanotechnology

2.1 Nanotechnology in the treatment of cancer

The past quarter century of outstanding progress in fundamental cancer biology has not translated into even distantly comparable advances in the clinic. Inadequacies in the ability to administer therapeutic moieties so that they will selectively reach the desired targets with marginal or no collateral damage has largely accounted for the discrepancy **(Langer 1998, Duncan 2003)**. Most striking is the recognition that only between 1 and 10 parts per 100,000 of intravenously administered monoclonal antibodies reach their parenchymal targets *in vivo* **(Li *et al.* 2004)**.

Nanotechnology concerns the study of devices that are themselves or have essential components in the 1–1,000 nm dimensional range (that is, from a few atoms to subcellular size)." (Ferrari M. 2005)

Similar limitations apply to contrast agents for imaging applications. There are two goals that should be kept in mind to increase the efficacy per dose of any therapeutic or imaging contrast formulation: firstly, to increase its targeting selectivity **(Allen 2002)** and second to endow the agent(s) comprising the therapeutic formulation with the means to overcome the biological barriers that prevent it from reaching its target **(Jain 1998)**. An ideal therapeutic system would be selectively directed against cell clusters that are in the early stages of the transformation towards the malignant phenotype. To make those goals achievable there are certain hurdles, those include: identification of suitable early markers of cancerous cells, and to understand their evolution over time. Tackling those issues could help nanotechnologist to overcome above stated issues for the site directed delivery of therapeutic and imaging agents.

Current problems and unmet needs in translational oncology include (a) advanced technologies for tumor imaging and early detection, (b) new methods for accurate diagnosis and prognosis, (c) strategies to overcome the toxicity and adverse side effects of chemotherapy drugs, and (d) basic discovery in cancer biology leading to new knowledge for treating aggressive and lethal cancer phenotypes such as bone metastasis. Understanding those basic parameters of cancer biology will help scientist to develop therapeutic and diagnosis modalities for the early detection and effective treatment of cancer.

What is cancer nanotechnology?

Cancer nanotechnology is new emerging multidisciplinary field that combines chemistry, engineering, physics, biology and medicine disciplines, and is expected to bring about revolution in cancer prognosis, diagnosis and treatment.

Nanoparticles are nano sized materials (diameter 1-1000 nm) that can carry multiple drugs and/or imaging agents. Owing to their high surface-area-to-volume ratio, it is possible to achieve high ligand density on the surface for targeting purposes. Nanoparticles can also be used to increase local drug concentration by carrying the drug within and control releasing it when bound to the targets. Currently, natural and synthetic polymers and lipids are typically used as drug delivery vectors. The family of nanoparticles includes polymer conjugates, polymeric nanoparticles, lipid-based carriers such as liposomes and micelles, dendrimers, carbon nanotubes, and gold nanoparticles, including nanoshells and nanocages (**Figure 1.5**). These nanocarriers have been explored for a variety of applications such as drug delivery, imaging, photo-thermal ablation of tumors, radiation sensitizers, detection of apoptosis, and sentinel lymph node mapping (**Duncan 2005, LaVan 2003**).

Nanocarriers can offer many advantages over free drugs. They:

- protect the drug from premature degradation;
- prevent drugs from prematurely interacting with the biological environment;
- enhance absorption of the drugs in a selected tissue (for example, solid tumor);
- control the pharmacokinetic and drug tissue distribution profile;
- improve intracellular penetration.

For rapid and effective clinical translation, the nanocarrier should:

- be made from a material that is biodegradable and biocompatible.
- well characterized, and easily functionalized.
- exhibit high differential uptake efficiency in the target cells over normal cells.
- be either soluble or colloidal under aqueous conditions;
- have an extended circulating half-life, a low rate of aggregation, and a long shelf life.

A. Nanowires

Nanowires are available in metallic, semiconductor, magnetic, oxide, and polymer compositions and are promising as ultra small chemical and biological sensors (**Wang *et al.* 2005**). Functionalized nanowires are coated with capture ligands such as antibodies or oligonucleotides. In the presence of target molecules, the specific binding between target molecule and capture molecule generates an immediate conductivity change within the nanowire that can be measured. Zheng *et al.* has developed nanowire arrays for multiplexed cancer biomarker detection (**Zheng *et al.* 2005**). Each nanowire of multiplexed system is coated with a distinct surface receptor capable of binding and sensing cancer biomarkers such as PSA, PSA-a1-antichymotrypsin, carcinoembryonic antigen, and mucin-1 in undiluted serum samples. These nanowire arrays allow simultaneous incorporation of control nanowires, which enables discrimination against false positives.

B. Cantilevers

Nanocantilevers-based sensors functions on the principal that biomolecular binding events results in a change in their resonating frequencies (**Fritz *et al.* 2000**). The nanoscale dimensions offer the possibility of designing structures with multiple such components, each with distinct detection capabilities, which can allow the simultaneous profiling of a range of markers for a given cancer. Furthermore, the reduction in the dimension also holds the promise of increasing the sensitivity to a scale where it is possible to detect subtle changes in the profile. Molecular probes, such as single-stranded DNA, can also be attached to beams just a few nanometers thick. When exposed to a DNA sample, complementary strands bind to the probes on the cantilever, causing the

beams to bend slightly. That response can be detected visually or by a change in the beams' electrical conductivity.

C. Nanobarcodes

An innovative approach for both protein and nucleic acid detection based on bio barcode-amplification (BCA) has been developed (**Nam *et al.* 2004**). This approach uses both colloidal gold nanoparticles and magnetic microbeads, in which gold nanoparticles are modified with both target capture strands and bar code strands that are subsequently hybridized to bar code DNA, and magnetic microparticles modified with target capture strands. In the presence of target DNA, the gold nanoparticles and the magnetic microbeads form sandwich structures that are magnetically separated from solution and are further washed to remove the unhybridized bar code DNA. The bar codes (hundreds to thousands per target) are detected by using a colorimetric method. This integrated capture and detection technology is four to six orders of magnitude more sensitive than standard ELISA (enzyme-linked immune sorbent assay) for proteins and offers comparable sensitivities as PCR (polymerase chain reactions) (**Nam *et al.* 2004**).

D. Quantum dots (QDs)

Semiconductor quantum dots (QDs) are tiny light-emitting particles on the nanometer scale that are emerging as a new class of fluorescent probes for in vivo biomolecular and cellular imaging (**Alivisatos 2004, Alivisatos 1996, Alivisatos *et al.* 2005, Pinaud *et al.* 2006, Michalet *et al.* 2005, Gao *et al.* 2005, Smith *et al.* 2004, Chan *et al.* 2002**) (**Figure 1.5**). They are made of inorganic elements such as cadmium or mercury encased in latex or metal and respond to light by emitting fluorescence at different wavelengths and intensities depending on their composition. Antibodies attached to the crystals can cause the dots to bind to a selective tissue, such as a tumor, which can then be more easily seen with conventional imaging devices. Also in comparison with organic dyes and fluorescent proteins, QDs have unique optical and electronic properties. QDs have molar extinction coefficients that are 10-50 times larger than that of organic dyes, which makes them much brighter in photon-limited *in vivo* conditions. Further, QD emission wavelengths are size-tunable. For example, CdSe/Zns QDs of approximately 2 nm in diameter produce a blue emission, whereas QDs approximately 7 nm in diameter emit red light (**Yu *et al.* 2003**). Recently scientists have extended the emission wavelength

into the near infrared (650 nm to 950 nm), to take advantage of the improved tissue penetration depth and reduced background fluorescence at these wavelengths **(Kim *et al.* 2005)**.

E. Carbon Nanotubes

Another type of nanodevice for biomarker detection is carbon nanotubes (CNTs) **(Wong *et al.* 1998)**. Using single-walled carbon nanotubes as high-resolution atomic force microscopy (AFM) tips, specific sequences of kilobase-size DNA can be selectively detected from single-base mismatch sequences **(Woolley *et al.* 2000)**. Specifically, target DNA fragments were first hybridized with labeled (for instance, strepavidin-labeled) oligonucleotides, and then AFM was used to directly detect the presence and specific location of the labels. This technique enabled the simple and direct detection of specific haplotypes that code for genetic disorders such as cancer. CNT-modified electrodes can amplify the electrochemical signal of guanuine bases. Those bases are used for label-free electrochemical detection of DNA at nanomolar concentrations **(Wang *et al.* 2004)**.

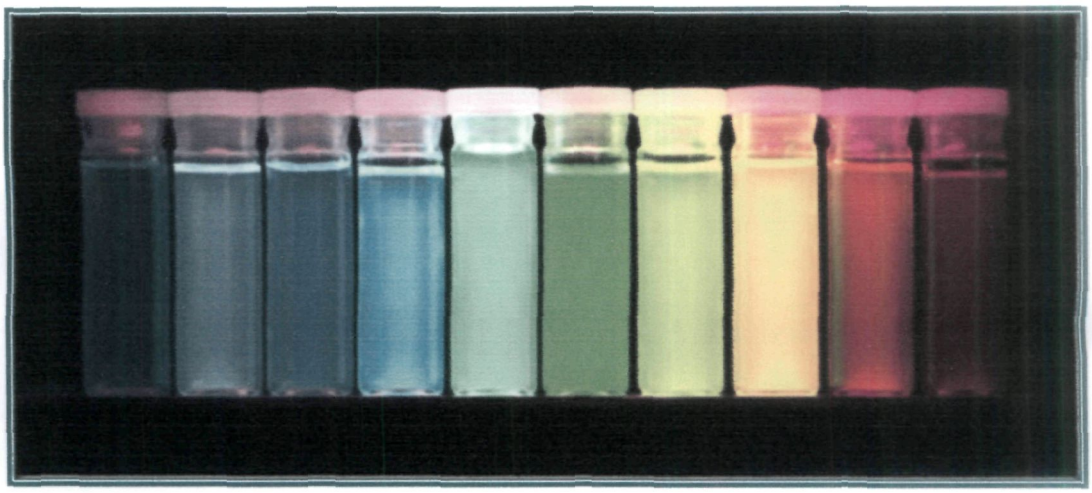


Figure 1.5 Semiconductor quantum dots with quantum confinement and size-tunable optical properties. This image shows ten distinguishable emission colors of ZnS-capped CdSe quantum dots excited with a near-UV lamp. From left to right (blue to red), the emission maxima are located at 443, 473, 481, 500, 518, 543, 565, 587, 610, and 655 nm **(adapted from Nie S *et al.* 2007)**.

F. Lipid-based nanocarriers

They have attractive biological properties, including general biocompatibility, biodegradability, isolation of drugs from the surrounding environment, and the ability to entrap both hydrophilic and hydrophobic drugs. Through the addition of agents to the lipid membrane or by the alteration of the surface chemistry, properties of lipid-based carriers, such as their size, charge, and surface functionality, can easily be modified. Liposomes, polymersomes, and micelles represent a class of amphiphile-based particles.

a) **Liposomes** are spherical, self-closed structures formed by one or several concentric lipid bilayers with inner aqueous phases. Today, liposomes are approved by regulatory agencies to carry a range of chemotherapeutics (**Gabizon 2001(a), Gabizon 2001(b). Safra** *et al.* **2000**).

b) **Polymersomes** have architecture similar to that of liposomes, but they are composed of synthetic polymer amphiphiles, including PLA-based copolymers (**Ahmed** *et al.* **2006, Discher** *et al.* **2006**). However, as with polymer therapeutics, there are still no clinically approved strategies that use active cellular targeting for lipid-based carriers.

c) **Micelles** are self-assembling closed lipid monolayers with a hydrophobic core and hydrophilic shell, have been successfully used as pharmaceutical carriers for water-insoluble drugs (**Matsumura** *et al.* **2004**). They belong to a group of amphiphilic colloids that can be formed spontaneously under certain concentrations and temperatures from amphiphilic or surface-active agents (surfactants) (**Figure 6a**). An example of a polymeric micelle under clinical evaluation is NK911, which is a block copolymer of PEG and poly aspartic acid. NK911, which consists of a bound doxorubicin fraction (~45%) (**Figure 6b**) and a free drug (**Kato** *et al.* **2006**), was evaluated for metastatic pancreatic cancer treatment. Another carrier NK105, a micelle containing paclitaxel, was evaluated for pancreatic, colonic and gastric tumor treatment (**Torchilin 2007**).

Lipid-based carriers pose several challenges, which represent general issues in the use of other targeted nanocarriers such as polymeric nanoparticles. For example, upon intravenous injection, particles are rapidly cleared from the bloodstream by the reticuloendothelial defense mechanism, regardless of particle composition (**Brigger** *et al.* **2002, Kreuter & Higuchi 1979**). Moreover, instability of the carrier and burst drug

release, as well as non-specific uptake by the mononuclear phagocytic system (MPS), provides additional challenges for translating these carriers to the clinic. Given their long history, liposome-based carriers serve as a classic example of the challenges encountered in the development of nanocarriers and the solutions that have been attempted. For example, PEG has been used to improve circulation time by stabilizing and protecting micelles and liposomes from opsonization - a plasma protein deposition process that signals Kupffer cells in the liver to remove the carriers from circulation (**Matsumura *et al.* 2004, Damascelli *et al.* 2001**). However, Daunosome and Myocet are examples of clinically used liposomes (80-90 nm in diameter) without PEG coating that have been reported to exhibit enhanced circulation times, although to a lesser degree than PEGylated liposomes such as Doxil/Caelyx.

G. Dendrimers

They are synthetic, branched macromolecules that form a tree-like structure and is a newly developed technology in the field of polymer chemistry. Polyamidoamine dendrimers have shown promise for biomedical applications because they (1) can be easily conjugated with targeting molecules, imaging agents, and drugs, (2) have high water solubility and well-defined chemical structures, (3) are biocompatible, and (4) are rapidly cleared from the blood through the kidneys, made possible by their small size (<5 nm), which eliminates the need for biodegradability. *In vivo* delivery of dendrimer-methotrexate conjugates using multivalent targeting results in a tenfold reduction in tumor size compared with that achieved with the same molar concentration of free systemic methotrexate (**Hong *et al.* 2007**). Although promising, dendrimers are more expensive than other nanoparticles and require many repetitive steps for synthesis, posing a challenge for large-scale production.

H. Nanoshells

Dual-modality and multifunctional probes are being developed by attaching molecular moieties with imaging, therapeutic, and targeting functions to nanostructured scaffolds. These integrated nanoparticle probes may allow simultaneous imaging and therapy of tumors and cardiovascular plaques in live animal models. Solid silica nanospheres, sometimes encased in a thin layer of gold, will travel through the bloodstream without entering most healthy tissues, but they tend to accumulate in tumor tissue. Therapeutic

molecules can be attached to the spheres, or once a large number of the nanoshells accumulate in a tumor. Depending on their composition, nanoshells can also absorb or scatter light, enhancing tumor images made with certain forms of spectroscopy. Nanoshells (100-200 nm) may use the same carrier for both imaging and therapy. They are composed of a silica core and a metallic outer layer. Nanoshells have optical resonances that can be adjusted to absorb or scatter essentially anywhere in the electromagnetic spectrum, including the near infrared region (NIR, 820 nm, 4 W cm^{-2}), where transmission of light through tissue is optimal. Absorbing nanoshells are suitable for hyperthermia-based therapeutics, where the nanoshells absorb radiation and heat up the surrounding cancer tissue. Scattering nanoshells, on the other hand, are desirable as contrast agents for imaging applications. Recently, a cancer therapy was developed based on absorption of NIR light by nanoshells, resulting in rapid localized heating to selectively kill tumors implanted in mice. Tissues heated above the thermal damage threshold displayed coagulation, cell shrinkage and loss of nuclear staining, which are indicators of irreversible thermal damage, whereas control tissues appeared undamaged (Hirsch *et al.* 2003, Loo *et al.* 2005).

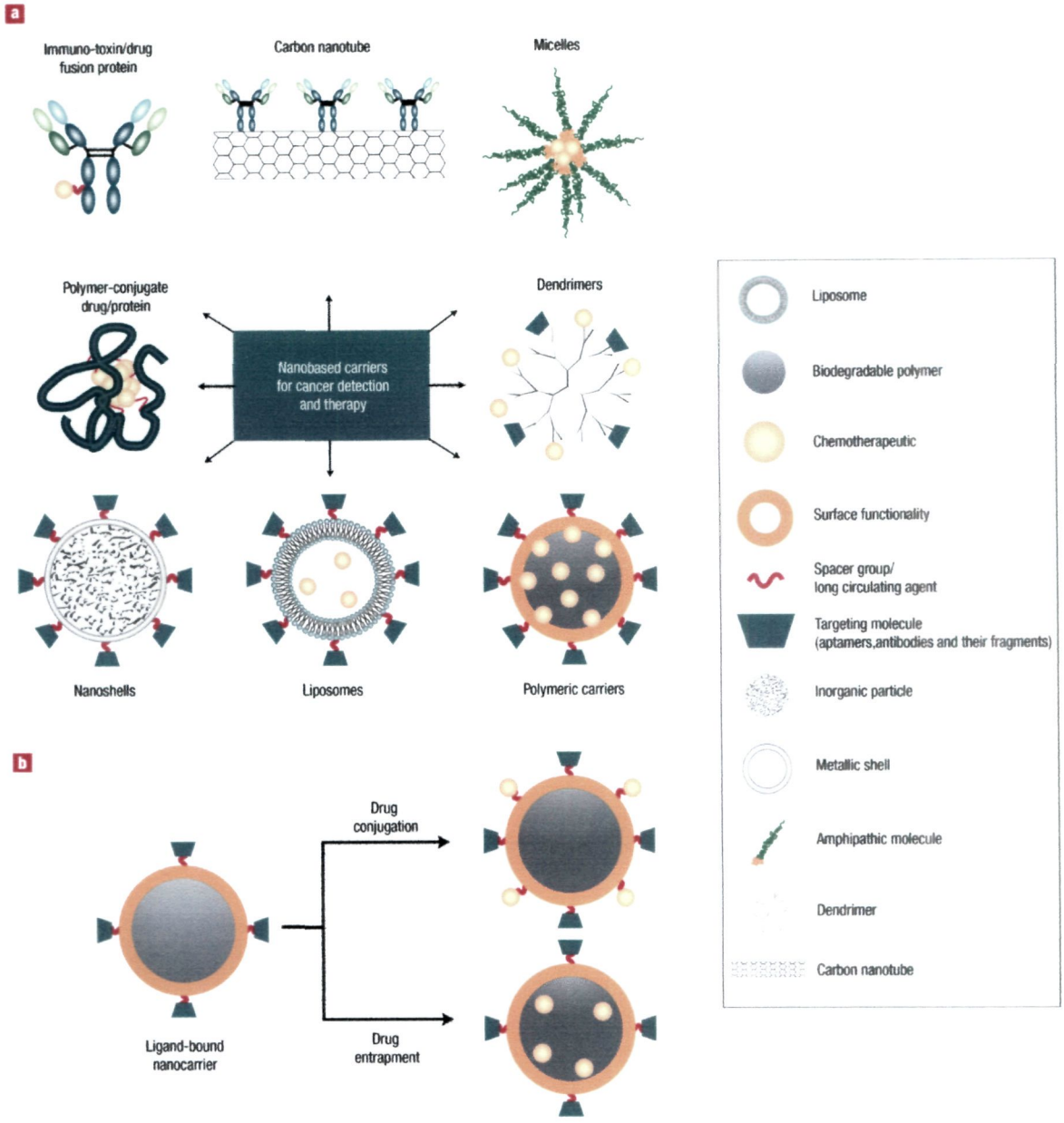


Figure 1.6 Nanocarriers for targeting cancer. A. A whole range of delivery agents are possible but the main components typically include a nanocarrier, a targeting moiety conjugated to the nanocarrier, and a cargo (such as the desired chemotherapeutic drugs). **B.** Schematic diagram of the drug conjugation and entrapment processes. The chemotherapeutics could be bound to the nanocarrier, as in the use of polymer drug conjugates, dendrimers and some particulate carriers, or they could be entrapped inside the nanocarrier **(adapted from Peer *et al.* 2007).**

I. Nanocages

A similar approach involves gold nanocages which are smaller (<50 nm) than the nanoshells. These gold nanocages can be constructed to generate heat in response to NIR light and thus may also be useful in hyperthermia-based therapeutics (**Chen *et al.* 2005**). Unlike nanoshells and nanocages, pure gold nanoparticles are relatively easy to synthesize and manipulate. Non-specific interactions that cause toxicity in healthy tissues may impede the use of many types of nanoparticles, but using inorganic particles for photo-ablation significantly limits non-specific toxicity because light is locally directed. However, inorganic particles may not provide advantages over other types of nanoparticles for systemic targeting of individual cancer cells because they are not biodegradable or small enough to be cleared easily, resulting in potential accumulation in the body, which may cause long-term toxicity.

J. Nanoparticles

Particles composed of a variety of materials can be constructed to contain therapeutic molecules in their core and to release them at a desirable time and location. Such delivery vehicles include simple lipid shells that passively leak through tumor blood vessel walls, and then slowly release a traditional chemotherapy drug into the tissue. Newer nanoparticles are more complexly designed, including exterior elements such as antibodies to target tumor-specific proteins, and materials that minimize the particles interaction with healthy tissues.

Inorganic nanoparticles are primarily metal based and have the potential to be produced with near monodispersity. Inorganic particles may also be functionalized to introduce targeting molecules and drugs. Specific types of recently developed inorganic nanoparticles include nanoshells and gold nanoparticles.

2.2 Application of nanotechnology in cancer therapy

Most current anticancer agents do not greatly differentiate between cancerous and normal cells, leading to systemic toxicity and adverse effects. Consequently, systemic applications of these drugs often cause severe side effects in other tissues (such as bone marrow suppression, cardiomyopathy, and neurotoxicity), which greatly limits the

maximal allowable dose of the drug. In addition, rapid elimination and widespread distribution into non-targeted organs and tissues requires the administration of a drug in large quantities, which is not economical and often complicated owing to non-specific toxicity. Nanotechnology offers a more targeted approach and could thus provide significant benefits to cancer patients. In fact, the use of nanoparticles for drug delivery and targeting is likely one of the most exciting and clinically important applications of cancer nanotechnology.

1. Passive Targeting

Rapid vascularization in fast-growing cancerous tissues is known to result in leaky, defective architecture and impaired lymphatic drainage. This structure allows an EPR (Enhanced Permeability and Retention) effect (**Matsumura & Maeda 1986, Duncan 2003, Jain 2001, Jain 1999**), resulting in the accumulation of nanoparticles at the tumor site (**Figure 1.7**). For such a passive targeting mechanism to work, the size and surface properties of drug delivery nanoparticles must be controlled to avoid uptake by the reticuloendothelial system (RES) (**Gref *et al.* 1994**). To maximize circulation times and targeting ability, the size should be optimized and the surface should be hydrophilic to circumvent clearance by macrophages. The hydrophilic surface of the nanoparticles safeguards against plasma protein adsorption and can be achieved through hydrophilic polymer coatings such as PEG, poloxamines, poloxamers, polysaccharides, or through the use of branched or block amphiphilic copolymers (**Ringsdorf 1975, Davis 2002, Moghimi & Hunter 2000, Park *et al.* 2005**). The covalent linkage of amphiphilic copolymers (polylactic acid, polycaprolactone, polycyanonacrylate chemically coupled to PEG) is generally preferred, as it avoids aggregation and ligand desorption when in contact with blood components.

An alternative passive targeting strategy is to utilize the unique tumor environment in a scheme called tumor-activated prodrug therapy. The drug is conjugated to tumor-specific molecule and remains inactive until it reaches the target (**Chari 1998**). Overexpression of the matrix metalloproteinase (MMP) MMP-2 in melanoma has been shown in a number of preclinical as well as clinical investigations (**Mansour *et al.* 2003**). Water-soluble maleimide derivative of doxorubicin (DOX) incorporating an MMP-2-specific peptide sequence (Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln), rapidly and selectively binds to the cysteine-34 position of circulating albumin. The albumin-DOX conjugate is efficiently

and specifically cleaved by MMP-2, releasing a DOX tetrapeptide (Ile-Ala-Gly-Gln-DOX) and subsequently DOX. pH and redox potential have been also explored as drug release triggers at the tumor site **(Guo & Szoka 2003)**. Another passive targeting method is the direct local delivery of anticancer agents to tumors. This approach has the obvious advantage of excluding the drug from the systemic circulation. However, administration can be highly invasive, as it involves injections or surgical procedures.

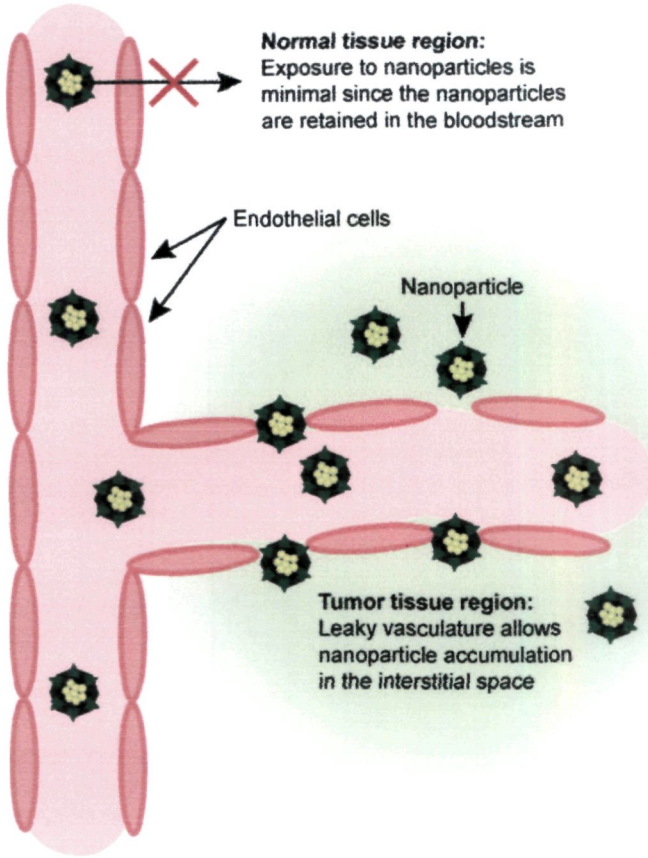


Figure 1.7 Enhanced permeability and retention of nanoparticles in tumors. Normal tissue vasculatures are lined by tight endothelial cells, thereby preventing nanoparticle drugs from escaping or extravasation, whereas tumor tissue vasculatures are leaking and hyperpermeable allowing preferential accumulation of nanoparticles in the tumor interstitial space (called passive nanoparticle tumor targeting) **(adapted from Nie *et al.* 2007)**.

2. Active Targeting

Active targeting is usually achieved by conjugating nanoparticle with a targeting component that provides preferential accumulation of nanoparticles in the tumor bearing organ, in the tumor itself, individual cancer cells, or intracellular organelles inside cancer cells. This approach is based on specific interactions, such as lectin carbohydrate, ligand-receptor, and antibody-antigen (**Allen 2002**). Lectin-carbohydrate is one of the classic examples of targeted drug delivery (**Kannagi *et al.* 2004**). Lectins are proteins of non-immunological origin, capable of recognizing and binding to glycoproteins expressed on cell surfaces. Lectin interactions with certain carbohydrates are very specific. Carbohydrate moieties can be used to target drug delivery systems to lectins (direct lectin targeting), and lectins can be used as targeting moieties to target cell surface carbohydrates (reverse lectin targeting). However, drug delivery systems based on lectin-carbohydrate have mainly been developed to target whole organs (**Yamazaki *et al.* 2000**), which can pose harm to normal cells. Therefore, in most cases the targeting moiety is directed towards specific receptors or antigens expressed on the plasma membrane or elsewhere at the tumor site. The overexpression of receptors or antigens in many human cancers lends itself to efficient drug uptake via receptor-mediated endocytosis (**Figure 1.8**). Because glycoproteins cannot remove polymer-drug conjugates that have entered the cells via endocytosis (**Bennis *et al.* 1994, Larsen *et al.* 2000**), this active targeting mechanism provides an alternative route for overcoming multiple drug resistance (MDR) (**Links & Brown 1999, Krishna & Mayer 2000, Vauthier *et al.* 2003, deVerdiere 1997, Blagosklonny 2003, Tsuruo 2003**). The cell surface receptor for folate is inaccessible from the circulation to healthy cells owing to its location on the apical membrane of polarized epithelia, but it is overexpressed on the surface of various cancers, including ovary, brain, kidney, breast, and lung malignancies (**Leamon & Reddy 2004, Leamon & Low 2001**). Surface plasmon resonance studies revealed that folate conjugated PEGylated cyanoacrylate nanoparticles had a tenfold higher affinity for the folate receptor than free folate did (**Stella 2000**). Folate receptors are often organized in clusters and bind preferably to the multivalent forms of the ligand. Furthermore, confocal microscopy demonstrated selective uptake and endocytosis of folate-conjugated nanoparticles by tumor cells bearing folate receptors. Interest in exploiting folate receptor targeting in cancer therapy and diagnosis has rapidly increased, as attested by many conjugated systems, including proteins, liposomes, imaging agents, and neutron activation compounds (**Leamon & Reddy 2004, Leamon & Low 2001, Stella *et al.* 2000**).

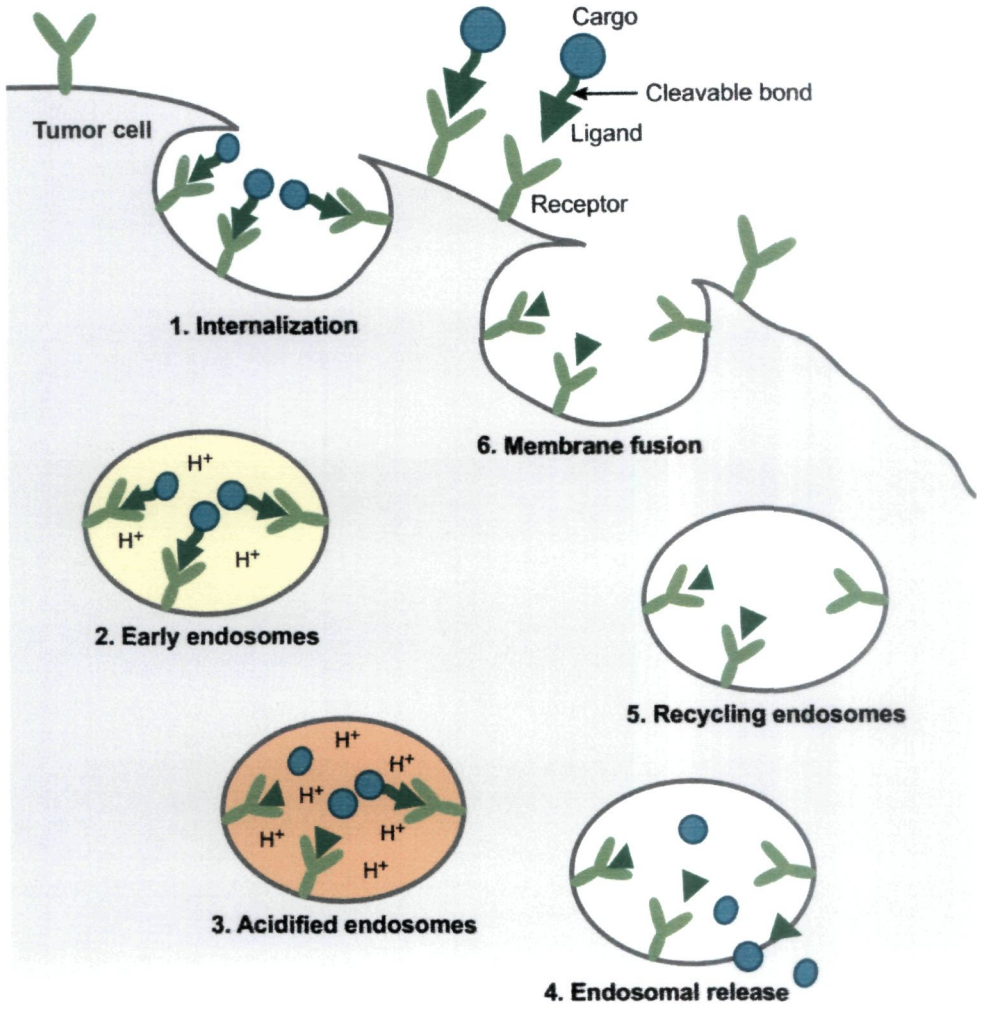


Figure 1.8 Nanoparticle based drug delivery and targeting using receptor-mediated endocytosis. The nanoparticle drug is internalized by tumor cells through ligand-receptor interaction. Depending on the design of the cleavable bond, the drug will be released intracellularly on exposure to lysosomal enzymes or lower pH **(adapted from Nie S *et al.* 2007)**.

3. Nanoparticle Drugs

Nanotechnology is beginning to change the scale and methods of drug delivery (**Figure 1.9**). Therapeutic and diagnostic agents can be encapsulated, covalently attached, or adsorbed onto nanoparticles. These approaches can easily overcome drug solubility issues, which have significant implications because more than 40% of active substances being identified through combinatorial screening programs are poorly soluble in water (**Merisko-Liversidge *et al.* 2003**). Conventional and most current formulations of such drugs are frequently plagued with problems such as poor and inconsistent bioavailability. The widely used attempt at enhancing solubility is to generate a salt. For non-ionizable compounds, micronization, soft-gel technology, co-solvents, surfactants, or complexing agents have been used (**Fishman *et al.* 2004**). Because it is faster and more cost effective to reformulate the drug than to develop a new one, a technology applicable to poorly water-soluble drugs could make a tremendous impact. For decades, researchers have been developing new anticancer agents and new formulations for delivering chemotherapeutic drugs (**Larsen *et al.* 2000**). Paclitaxel (Taxol TM) is one of the most widely used anticancer drugs in the clinic. It is a microtubule-stabilizing agent that promotes tubulin polymerization, disrupting cell division and leading to cell death (**Diaz *et al.* 2000, Nicolaou *et al.* 1993**). It displays neoplastic activity against primary epithelial ovarian carcinoma and breast, colon, and lung cancers. Because it is poorly soluble in aqueous solution, the formulation available currently is Chremophor EL (polyethoxylated castor oil) and ethanol (**Singla *et al.* 2002**). In a new formulation approach used in Abraxane TM, recently approved by the FDA to treat metastatic breast cancer, paclitaxel was conjugated to albumin nanoparticles (**Garber 2004**). The formulation is very effective in circumventing side effects of the highly toxic Chremophor EL, which includes hypersensitivity reactions, nephrotoxicity, and neurotoxicity (**Singla *et al.* 2002, Gelderblom *et al.* 2001**). Although the SPACR (secreted protein, acidic, cysteine-rich, also called osteonectin) protein is believed to improve albumin drug uptake, this nanoparticulate drug still exhibits significant side effects (see FDA-Approved Nanoparticle Drug-Abraxane).

For enhanced tumor-specific targeting, the differences between cancerous cells and normal cells may be exploited. By virtue of their small size, nanoparticles entail a high surface area that not only paves the way for more efficient drug release but also a better strategy for functionalization. There is a growing body of knowledge of unique cancer markers, thanks to recent advances in proteomics and genomics. They form the basis of

complex interactions between bio-conjugated nanoparticles and cancer cells. Carrier design and targeting strategies may vary according to the type, developmental stage, and location of cancer **(Vicent & Duncan 2006)**.

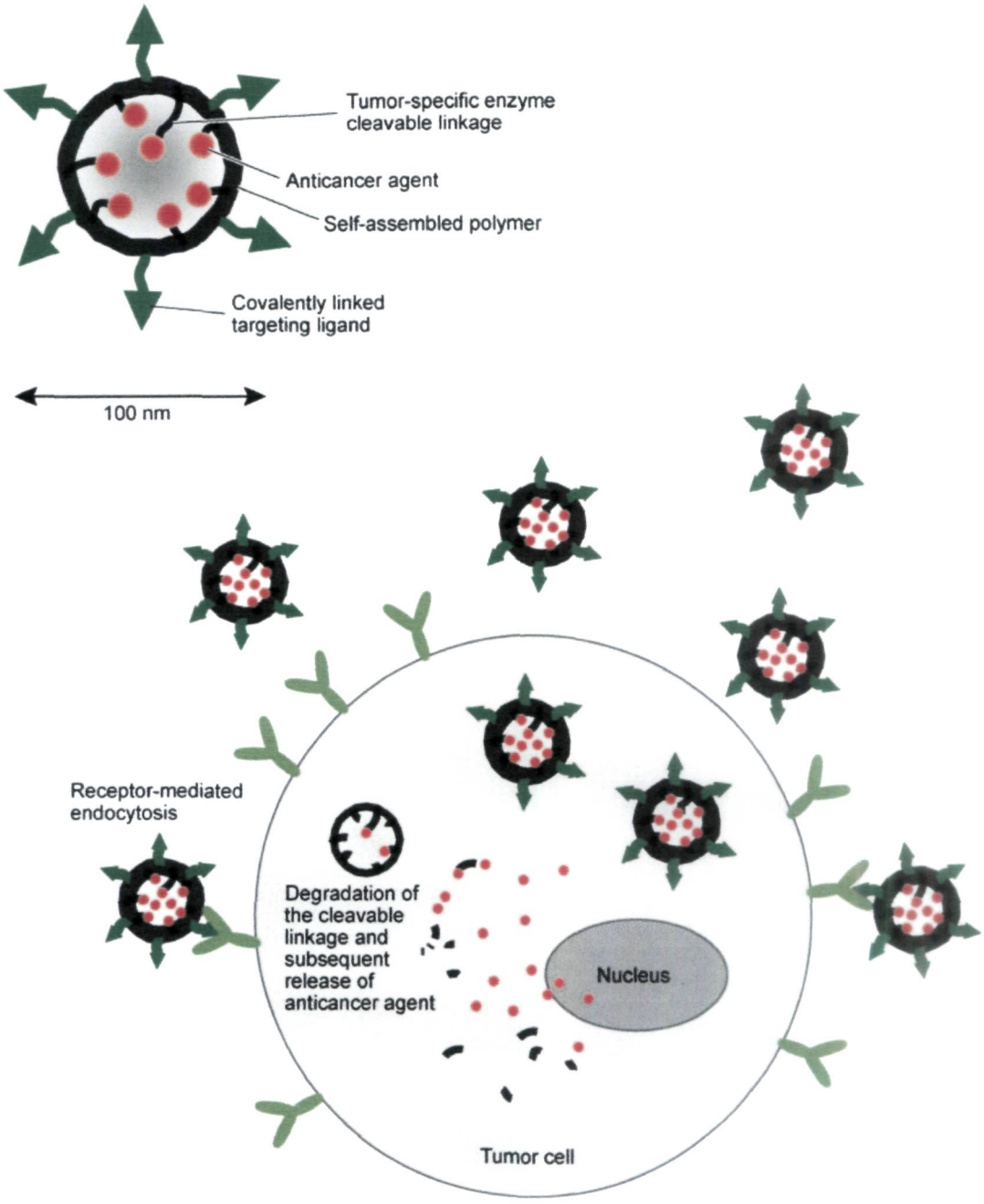


Figure 1.9 Self-assembled polymeric nanoparticles with dual tumor-targeting and therapeutic functions *(upper panel)* and delivery of the nanoparticle drugs by receptor-mediated endocytosis and controlled drug release inside the cytoplasm *(lower panel)* **(adapted from Nie S *et al.* 2007)**.

There is much synergy between imaging and nanotechnology in biomedical applications. Many of the principles used to target delivery of drugs to cancer may also be applied to target imaging and diagnostic agents to enhance detection sensitivity in medical imaging. With engineered multifunctional nanoparticles **(Figures 1.10)**, the full *in vivo* potential of cancer nanotechnology in targeted drug delivery and imaging can be realized.

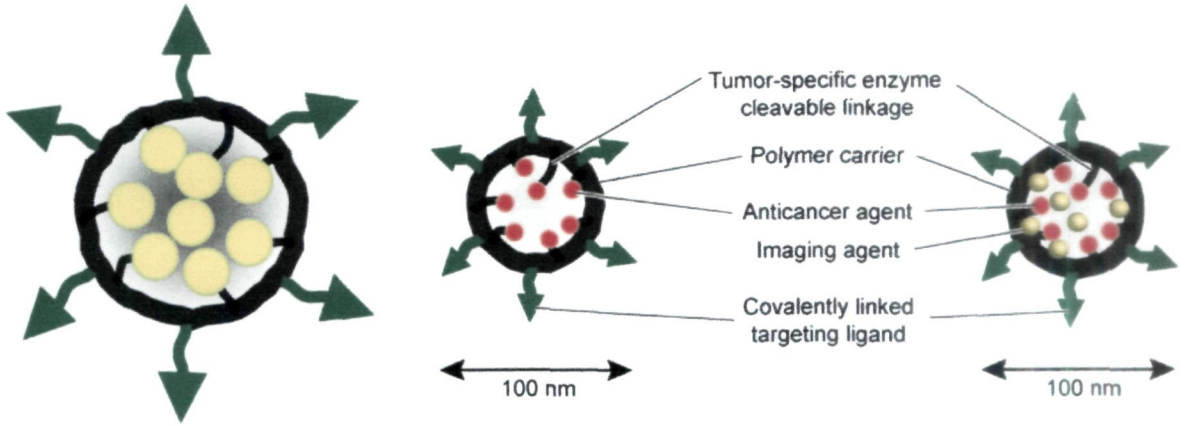


Figure 1.10 Multifunctional nanoparticles for integrated cancer imaging and therapy. A truly exciting feature of cancer nanotechnology is that drug delivery, treatment efficacy, and toxicity could be monitored by using embedded imaging agents **(adapted from Nie S *et al.* 2007)**.

The medical application of nanotechnology (that is, 'nanomedicine') has enormous potential to improve healthcare, particularly in cancer **(NCI Plan. 2004, European Science Foundation. 2005, Ferrari 2005)**. On one hand, miniaturization is creating devices for use as diagnostics, biosensors and imaging agents, and on the other, ever more sophisticated synthetic chemistry is producing nanovectors for drug delivery. The terminology used is often contentious and can be confusing. Ferrari recently coined a useful definition of cancer nanotechnology **(Ferrari 2005)** as "a vast and diverse array of devices derived from engineering, biology, physics and chemistry, including nanovectors for the targeted delivery of anticancer drugs and imaging contrast agents, and those detection systems such as nanowires and nanocantilever arrays under development for the early detection of precancerous and malignant lesions from biological fluids." Nanovectors have also been called 'nanopharmaceuticals or nanomedicines'. To distinguish them from biotech products, such as proteins and antibodies (which are also inherently 2-15 nm in size), the European Science Foundation's Forward Look on

Nanomedicine defined nanomedicines as "**nanometer size scale complex systems, consisting of at least two components, one of which being the active ingredient**" (**European Science Foundation. 2005**).

4. Polymer therapeutics as anticancer agents

Natural and synthetic polymers are used widely as components of new medical devices, for example, as rate-controlling coatings, as hydrogels or matrices for the topical administration of drugs, in tablets and capsules for oral administration and controlled release systems for drugs, peptides and proteins, and as constructs for tissue engineering. However, it has only been during the last decade that the first polymer-based therapeutics emerged as clinically accepted medicines for parenteral administration. The term 'polymer therapeutics' was coined to describe the biologically active polymeric drugs (**Donaruma 1974, Seymour 1991, Regelson & Parker 1986**), polymer–drug conjugates (**Duncan 1992, Duncan 2003, Kopecek *et al.* 2000**), polymer–protein conjugates (**Harris & Chess 2003, Pasut *et al.* 2004**), polymeric micelles to which a drug is covalently bound (**Yokoyama *et al.* 1990**) and multi-component polyplexes (containing covalent linkers) being developed as non-viral vectors for gene and protein delivery (**Pack 2005, Wagner & Kloeckner 2005**). From the industrial standpoint, these are new chemical entities rather than conventional drug-delivery systems or formulations that simply entrap, solubilize or control drug release without resorting to chemical conjugation. The distinction is between a covalently bound biologically active system, and one that is non-covalently complexed or simply entrapped.

Polymer-drug conjugates

Biological rationale for the design of polymer–anticancer drug conjugates and methods for their preclinical evaluation (**Kopecek *et al.* 2000, Duncan 2005**) still hold true today. The clinical aims of polymer-drug conjugation are, to achieve improved drug targeting to the tumor, to reduce drug toxicity (by limiting access to the sites of toxicity) and to overcome the mechanisms of drug resistance. First generation conjugates sought to improve the therapeutic index of drugs already in routine clinical use (for example, doxorubicin, camptothecins, paclitaxel and platinates including carboplatin and diaminocyclohexane (DACH)-platinates). Conjugation to hydrophilic polymeric carriers can also improve the water solubility of hydrophobic drugs such as doxorubicin and

paclitaxel, enabling easier formulation and patient administration. Almost all polymer-drug conjugates that have been clinically tested rely on increased tumor vascular permeability (EPR effect) for tumor targeting. Many preclinical studies suggest opportunities for the tumor-specific targeting of polymer conjugates (**Duncan & Izzo 2005, Duncan 2005**) using antibodies, peptides (example, melanocyte stimulating hormone (**O'Hare *et al.* 1993**) and other ligands (example, folate (**Low & Antony 2004**); only one such targeted conjugate, HPMA copolymer-doxorubicin-galactosamine has so far been evaluated clinically. Many polymers have been proposed as drug carriers (**Brocchini & Duncan 1999**), but few have progressed to *in vivo* or clinical studies. Problems that are prohibiting further development include inherent polymer-related toxicity and/or polymer-related immunogenicity (polymers, as macromolecules, can cause an immune response (unlike PEG), so choice of polymer should be made carefully (**Rihova 1996**); inadequate drug loading or inappropriate choice of drug (usually potency too low); and the use of unsuitable polymer- drug linkers - being either too stable (therefore preventing drug liberation and access to the pharmacological target), or degrading too quickly in aqueous solutions leading to premature drug release.

Design of polymer-drug conjugates

Induction of polymer chemistry for drug conjugation (**Ringsdorf 1975**) and later demonstration of the endocytic pathway useful for lysosomotropic drug delivery (**de Duve *et al.* 1974**) led to the concept of targetable anticancer polymer-drug conjugates. Low-molecular-weight anticancer agents typically distribute randomly throughout the body, and this often leads to side effects. The attachment of drugs to polymeric carriers can:

- Limit cellular uptake to the endocytic route.
- Produce long-circulating conjugates.

Most of the dose of low-molecular-weight drug typically leaves the circulation within minutes, whereas a polymer conjugate will ideally circulate for several hours to facilitate passive tumor targeting caused by the leakiness of angiogenic tumor blood vessels by the enhanced permeability and retention effect (EPR effect) (**Matsumura & Maeda 1986**). Conjugates have also been synthesized to contain targeting ligands (such as antibodies,

peptides and sugars) with the aim of further promoting increased (building on the EPR effect) tumor targeting by receptor-mediated delivery (**Duncan 1992**).

Several features are needed for the effective design of polymer–drug conjugates:

- The polymer must be non-toxic and non-immunogenic. It must also be suitable for industrial-scale manufacture. Polymer molecular weight should be high enough to ensure long circulation, but for non-biodegradable polymeric carriers this molecular weight (Mw) must be less than 40,000 g mol^{-1} to enable the renal elimination of the carrier following drug delivery. Therefore, the optimum (usually Mw 30,000-100,000 g mol^{-1}) must be tailored to suit the particular polymer being used.
- The polymer must be able to carry an adequate drug payload in relation to its potency.
- The polymer-drug linker must be stable during transport to the tumor, but able to release the drug at an optimum rate on arrival within tumor cells.
- If the drug exerts its effects through an intracellular pharmacological receptor, access to the correct intracellular compartment is essential. Peptidyl and ester polymer-drug linkers have been widely used. In particular, peptide sequences are designed that undergo cleavage by the lysosomal thiol-dependent protease cathepsin B (**Duncan *et al.* 1983**), but often pH-sensitive *cis*-aconityl, hydrazone and acetal linkages have also been used which are hydrolysed within endosomal and lysosomal vesicles because of the local acidic pH (6.5-4.0). The ideal rate of release will vary according to the mechanism of action of the drug being delivered. Typically, conjugates containing doxorubicin linked by Gly-Phe-Leu-Gly release the drug payload over 24-48 hr.
- The intracellular delivery and transfer of a drug out of the endosomal or lysosomal compartment is in many cases not only essential for therapeutic activity (**Lloyd 2001**), it also provides the opportunity to bypass mechanisms of drug resistance that are reliant on membrane efflux pumps such as p-glycoprotein (**Minko *et al.* 1998**). The limitation of polymer Mw to <100,000 g mol^{-1} ensures that the conjugate will be small enough to extravasate easily into the tumor, and will enable endocytic internalization by all types of tumor cells.

2.3 Mechanism of action of nanoparticles

a. Drug that is covalently bound by a linker that is stable in the circulation is largely prevented from accessing normal tissues (including sites of potential toxicity), and bio-distribution is initially limited to the blood pool.

b. The blood concentration of drug conjugate drives tumor targeting due to the increased permeability of angiogenic tumor vasculature (compared with normal vessels), providing the opportunity for passive targeting due to the enhanced permeability and retention effect (EPR effect).

c. Through the incorporation of cell-specific recognition ligands it is possible to bring about the added benefit of receptor-mediated targeting of tumor cells.

d. It has also been suggested that circulating low levels of conjugate (slow drug release) might additionally lead to immunostimulation.

e. If the polymer-drug linker is stable in the circulation, for example, *N*-(2-hydrox ypropyl) methacrylamide (HPMA) copolymer-Gly-Phe-Leu-Gly-doxorubicin, the relatively high level of renal elimination (whole body t1/2 clearance >50% in 24 h) compared with free drug (t1/2 clearance ~50% in 4 days) can increase the elimination rate.

f. On arrival in the tumor interstitium, polymer-conjugated drug is internalized by tumor cells through either fluid-phase pinocytosis (in solution), receptor-mediated pinocytosis following non-specific membrane binding (due to hydrophobic or charge interactions) or ligand–receptor docking. Depending on the linkers used, the drug will usually be released intracellularly on exposure to lysosomal enzymes (for example, Gly-Phe-Leu-Gly and polyglutamic acid (PGA) are cleaved by cathepsin B) or lower pH (for example, a hydrazone linker are degraded in endosomes and lysosomes (pH 6.5- <4.0).

The active or passive transport of drugs such as doxorubicin and paclitaxel out of these vesicular compartments ensures exposure to their pharmacological targets. Intracellular delivery can bypass mechanisms of resistance associated with membrane efflux pumps such as p-glycoprotein. If >10-fold, EPR-mediated targeting will also enable the circumvention of other mechanisms of drug resistance. Non-biodegradable polymeric platforms must eventually be eliminated from the cell by exocytosis. Rapid exocytic elimination of the conjugated drug before release would be detrimental and prevent access to the therapeutic target. In general, polymeric carriers do not access the cytosol.

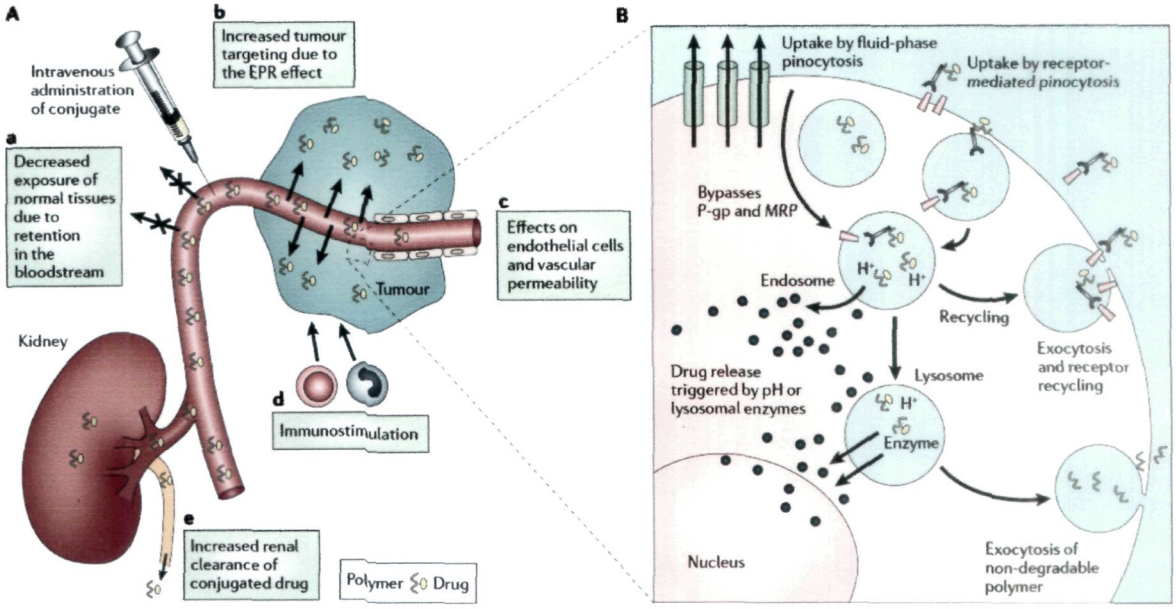


Figure 1.11 Understanding of the mechanism of action of polymer-drug conjugates. Hydrophilic polymer drug conjugates administered intravenously can be designed to remain in the circulation - their clearance rate depends on conjugate molecular weight, which governs the rate of renal elimination **(adapted from Duncan R 2006)**.

Part III:
siRNA based cancer therapy

3.1 siRNA based cancer therapy

Antisense technology has expanded to an outstanding limit since its initial discovery in the nematode *Caenorhabditis elegans* (**Fire *et al.* 1998**). Soon after this, Elbashir SM *et al.* showed that double stranded interfering RNAs (siRNAs) of ~21 nt in length bring about gene silencing in mammalian cells (**Elabashir *et al.* 2001**). Followed by discovery, knowledge of its mechanism of action brings new hope for therapeutic interventions of human diseases. The key therapeutic advantage of using RNAi lies in its ability to specifically and potently knock down the expression of disease-causing genes of known sequence. Furthermore, the relatively short turnaround time for efficacy testing of potential therapeutic RNAi molecules, and the fact that even newly discovered pathogens are theoretically amenable to rapid targeting, has caused great excitement about the potential of RNAi for treating a wide range of diseases.

Consistent research is going on to utilise siRNA as a therapeutic in various disease caused by gene disruption, results of which have spurred cautious optimism about the promise of RNAi-based therapies. Very first demonstration of clinical applications of RNAi have been directed at the treatment of wet, age-related macular degeneration (AMD) (**Check *et al.* 2005**) and respiratory syncytial virus (RSV) infection (**Bitko *et al.* 2005**). Therapies based on RNAi are also in preclinical development for other viral diseases (**Rossi 2006, Dykxhoorn & Lieberman 2006**) neurodegenerative disorders (**Raoul *et al.* 2006**) and cancers (**Pai *et al.* 2006**), although a number of challenges need to be addressed and improvements made for RNAi-based therapies to realize their full potential. A progressively more detailed understanding of the basic mechanisms of RNAi has been important in developing diverse RNAi effector molecules with improved levels of potency and efficacy.

For example, synthetic siRNAs and expressed short hairpin RNAs (shRNAs) (**Amarzguioui *et al.* 2005**) both have specific advantages and disadvantages, which are important considerations when designing RNAi-based therapies for a particular disease. In addition, although many *in vivo* studies have shown the potential effectiveness of various RNAi-based strategies, other studies have highlighted challenges that arise as a result of using an endogenous cellular mechanism for therapeutic benefit. Unwanted side effects have included induction of type 1 interferon (IFN) responses (**de Veer *et al.* 2005**) and saturation of endogenous RNAi pathway components (**Grimm *et al.* 2006**),

indicating that caution is necessary when designing effector molecules for delivery into target cells. The issue of cell-specific or tissue-specific delivery is another key challenge in developing RNAi based therapies. Various strategies for non-viral and viral delivery of RNAi triggers have recently been shown to be effective in disease models, raising the hope that clinical studies of RNAi-based therapies will be extended to an increasing list of diseases in the near future (**Behlke 2006**).

3.2 Mechanisms of RNAi-mediated gene silencing

Gene silencing by RNAi is guided by two pathways: a) siRNA b) microRNAs (miRNAs). RNAi complements with its homologous sequence that brings about its translational repression and transcript degradation. RNAi also blocks gene expression by direct transcriptional gene silencing (TGS) in the nucleus although its mechanism of action is not yet clear in mammalian systems (**Figure 1.12**).

a) **siRNA mediated silencing:** Briefly, double stranded RNA (dsRNA) is cleaved into small interfering RNA by a complex containing Dicer, TAR RNA-binding protein (TRBP) and protein activator of protein kinase PKR (PACT). Those siRNAs are loaded into Argonaute 2 (AGO2) and the RNA-induced silencing complex (RISC). Now catalytic domain of AGO2 recognizes target sites in mRNA and brings about its cleavage. siRNAs complementary to promoter regions direct transcriptional gene silencing in the nucleus through chromatin changes involving histone methylation; the precise molecular details of this pathway in mammalian cells are currently unclear.

b) **microRNA mediated silencing:** As shown in **figure 1.12**, endogenously encoded primary microRNA transcripts (pri-miRNAs) are transcribed by RNA polymerase II (Pol II) and initially processed by Drosha–DGCR8 (Di George syndrome critical region gene 8) to generate precursor miRNAs (pre-miRNAs). These precursor miRNAs are exported to the cytoplasm with the help of exportin 5 and subsequently bind to the Dicer-TRBP-PACT complex, which processes the pre-miRNA for loading into AGO2 and RISC. The mature miRNA recognizes target sites in the 3' untranslated region (3' UTR) of mRNAs to direct translational inhibition and mRNA degradation in processing (P)-bodies that contain the decapping enzymes DCP1 and DCP2.

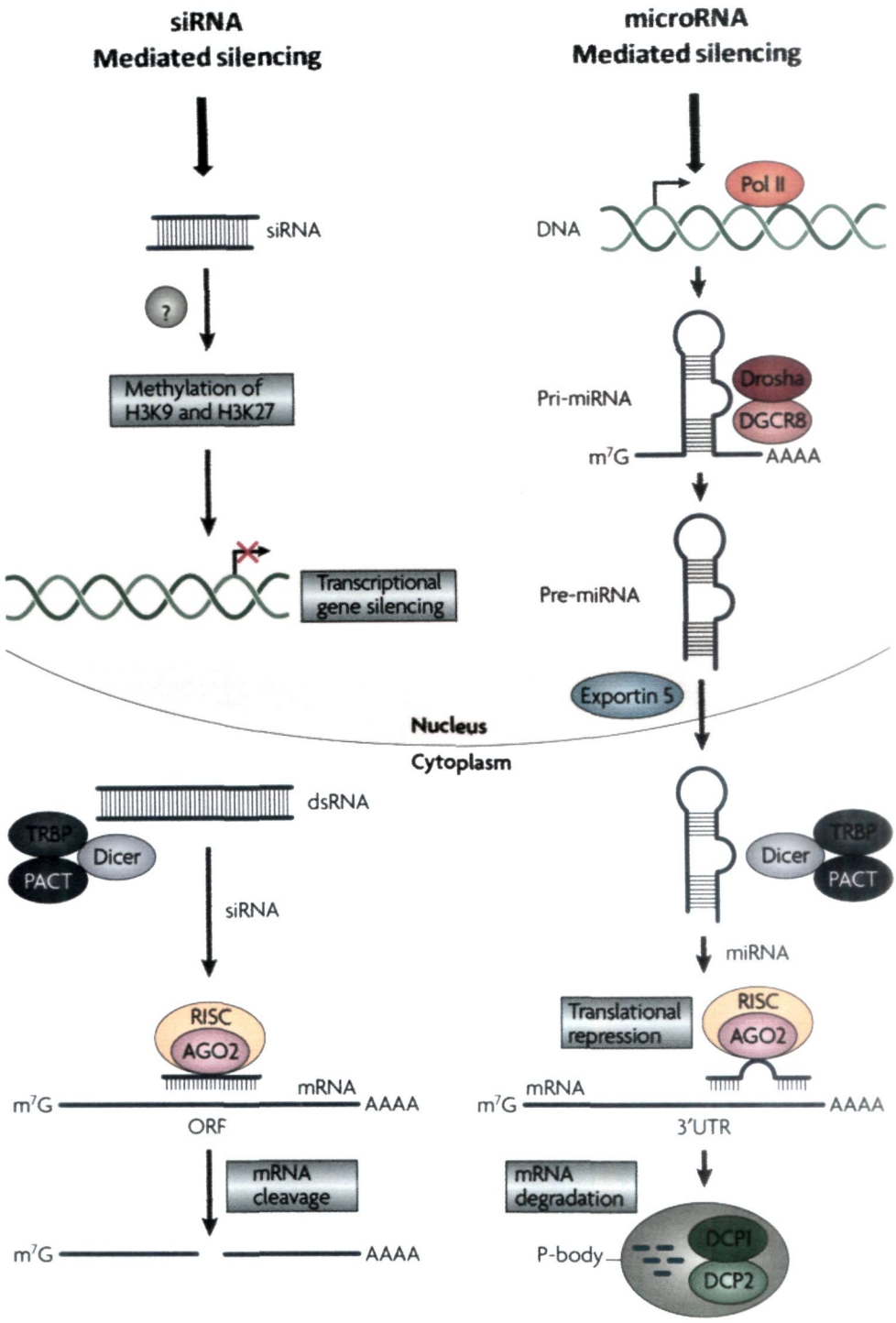


Figure 1.12 Mechanisms of RNA interference in mammalian cells (Adapted from Kim DH and Rossi JJ 2007).

Challenges of siRNA therapy

siRNA technology has begun to be viewed as new hope for the treatment of genetic disorders, infections and cancer. It certainly offers potential advantages but there are certain issues to be addressed. One of which is "off target" effect; this is the inhibition of a gene, the expression of which should not be targeted, because the gene shares partial homology with the siRNA. The inadvertent silencing of non-target genes may lead to problems in interpretation of data and potential toxicity. To avoid this issue, we should design and select target very carefully. The basic parameters for chosing siRNAs involve consideration of internal repeated sequences, secondary structure, GC content, base preference at specific positions in the sense strand, and appropriate siRNA length (19-22 bps). To predict off-target effects for an siRNA sequence, several computer algorithms have been developed. For example, AsiDesigner is a freely accessible web tool (http://sysbio.kribb.re.kr/AsiDesigner/), which provides stepwise off-target searching with BLAST and FASTA algorithms (**Park** *et al.* 2008).

Another challenge facing siRNA therapy is 'immune stimulation'; this is recognition of an siRNA duplex by the innate immune system (**de Fougerolles** *et al.* 2007, **Bumcrot** *et al.* 2006). Introduction of too much siRNA activates innate immune responses. The immune system is probably activated via the dsRNA sensor, protein kinase R. Inflammatory cytokines and interferons were found to be induced by activation of NF-kB and interferon regulatory factors following the recognition of siRNA by toll-like receptor 7 (TLR7), TLR8, and TLR9 (**Marques** *et al.* 2005). Recently, 21-nucleotide or longer siRNAs were reported to activate TLR3, a double-stranded viral RNA sensor, and suppress neovascularization in sequence and target-non specific manner (**Kleinman** *et al.* 2008).

However, it has been reported that not all siRNAs could induce immune stimulation (**Judge** *et al.* 2005, **Hornung** *et al.* 2005) and stimulation of innate immune response is nucleotide sequence dependent (**Judge** *et al.* 2005). The TLR7-mediated interferon alpha induction by siRNA was shown to be sequence specific (**Hornung** *et al.* 2005). The last, but the most important, challenge in siRNA therapy is the issue of delivery. Being an RNA entity, siRNA is anionic, hydrophilic, and unable to enter cells by passive diffusion mechanisms. Moreover, *in vivo* delivery of naked siRNA to appropriate disease sites remains a considerable hurdle owing to rapid enzymatic digestion in plasma and

renal elimination, limited penetration across the capillary endothelium, and inefficient uptake by tissue cells (**Bumcrot *et al.* 2006**). To overcome these difficulties, the development of effective *in vivo* delivery systems is essential.

3.4 siRNA delivery systems for in vivo application

Keeping in view the hurdles that affect siRNA delivery researchers have developed many vectors for *in vitro* delivery of siRNA but still we need to come up with a suitable delivery vector for *in vivo* applications (**Bumcrot *et al.* 2006**). Currently, siRNAs in clinical trials are directly administered to local target sites such as the eye and lung, thereby avoiding the complexity of systemic delivery. However, it is necessary to introduce siRNA by a systemic route to treat most cancers and other diseases.

The optimal *in vivo* systemic delivery systems for siRNA should be biocompatible, biodegradable, and non-immunogenic. Second, the systems should provide efficient delivery of siRNA into target cells or tissues with protection of the active double-stranded siRNA products from attack by serum nucleases. Next, the delivery systems must provide target tissue-specific distribution after systemic administration, avoiding rapid hepatic or renal clearance. Finally, after delivery into target cells via endocytosis, the systems should promote the endosomal release of siRNA into the cytoplasm, allowing the interaction of siRNA with the endogenous RISC (**Juliano *et al.* 2008, Aigner 2007**).

To confer drug-like properties such as stability, cellular delivery, and tissue bioavailability to siRNAs, various strategies that range from chemical modification of siRNA to design of different non-viral vectors have been developed and validated.

1. Chemical modification

To enhance the stability of siRNA for prolonged circulation in vivo, chemical modification of siRNA has been attempted. Various positions within the siRNA duplex have been chemically replaced or modified to provide nuclease resistance. One of the common approaches is replacement of the phosphodiester (PO_4) group with phosphothioate (PS) at the 3'-end. Moreover, the introduction of an O-methyl group (2'-O-Me), a fluoro (2'-F) group, or a 2-methoxyethyl (2'-O-MOE) group resulted in prolonged half-lives and RNAi activities in cultured cells and plasma (**Chiu *et al.* 2003,**

Harborth *et al.* 2003, Czauderna *et al.* 2003, Braasch *et al.* 2003, Layzer *et al.* 2004). Modification of siRNA using small molecules such as 2,4-dinitrophenol (DNP) was found to increase not only nuclease resistance but also the membrane permeability of siRNA (Iiao & Wang 2005) Indeed, boranophosphonate siRNA showed a better resistance to nuclease degradation than did an unmodified siRNA, but boranophosphonate modification at the central position of the antisense strand reduced RNAi activity (Hall *et al.* 2004). In addition, the degradation of a modified siRNA into molecules not natural to the body may give rise to the fear that metabolites of such an siRNA may be unsafe.

2. Lipid-based siRNA delivery

Various lipid-based delivery systems have been developed for *in vivo* application of siRNA. Lipid-based systems include liposomes, micelles, emulsions, and solid lipid nanoparticles. For the delivery of siRNA using lipid-based systems, lipid composition, drug-to lipid ratio, particle size, and the manufacturing process should be optimized. Among synthetic delivery systems for siRNA, cationic liposomes have emerged as one of the most attractive vehicles owing to the simple manner in which such liposomes form complexes with negatively charged siRNA, their high transfection efficiency, their enhanced pharmacokinetic properties, and their relatively low toxicity and immunogenicity. Moreover, cationic liposomes can protect siRNA from enzymatic degradation, and provide reduced siRNA renal clearance.

3. Polymer-mediated siRNA delivery

Polymer-based delivery systems have been extensively used for plasmid DNA and more recently for siRNA. As with lipid-based delivery systems, polymeric delivery of siRNA usually involves a cationic moiety as a core component. The development since 1990 of cationic polymers for delivery of plasmid DNA has resulted in the accumulation of much relevant information. siRNA differs from plasmid DNA in molecular weight, charge ratio, stability, and method of action. However, as both are nucleic acids, siRNA and plasmid DNA share a number of characteristics relevant to in vivo delivery. Accordingly, polymer-mediated DNA delivery systems would provide a lot of informative knowledge for the development of polymer-based siRNA therapeutics (Gary *et al.* 2007).

Chapter 2:

Anticancer efficacy of perillyl alcohol-bearing PLGA microparticles

Cytotoxicity and other related side effects are the most serious problems associated with the currently available anticancer drugs. Other limitations include widespread systemic distribution and rapid elimination of the administered anticancer drugs from the host body. A worldwide search therefore continues for anticancer drugs that are more potent, less toxic, and manifest minimum untoward effects to the host. Several plant derived compounds have been reported to possess strong anticancer properties and have been shown to delay, inhibit, or reverse cancerous growth in an effective manner. For example, perillyl alcohol (POH), a plant-based compound, has been reported to possess strong anti-cytotoxic properties against several types of cancer including those of breast, pancreatic, and liver (**Haag & Gould 1994, Stark *et al.* 1995**). POH is a monoterpene and constituent of essential oils from a number of plants; namely, perilla (*Perilla frutescens*), lavendin, peppermint, ginger grass, savin, caraway, and celery seeds (**Figure 2.1**).

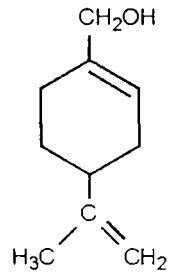


Figure 2.1 Chemical structure of perillyl alcohol.

Before translating the suitability of a novel compound like POH as a potential anticancer agent in the clinical setting, it is desirable to address some of the associated issues like that of its solubility, palatability, and sustained/controlled release in systemic circulation. This requires designing of a suitable drug-delivery system that can release the drug gradually over a long period of time and, in turn, facilitate its uptake by cancer cells and thereby helps in increasing the efficacy of the entrapped drug. Polymeric microparticles offer a promising technology in this regard.

Encapsulation of drugs in the core of microparticles have been shown not only to protect them from the external environment enroute, but also help in increasing their plasma half-lives in systemic circulation thereby facilitating the attainment of optimum drug availability at the desired target (**Muller *et al.* 2002, Spiclin *et al.* 2003, Fang *et al.* 2006**).

To develop promising and effective formulations of anticancer compounds, various polymeric matrices have been investigated and their efficacies worked out.5–10 Drug delivery systems, such as nanoparticle (**Muller *et al.* 2002**), microemulsion (**Spiclin *et al.* 2003**), nanoemulsion (**Fang *et al.* 2006**), and liposome-based delivery systems (**Betz *et al.* 2005, Kirjavainen *et al.* 1999, Khan *et al.* 2007**), have been shown to enhance the efficacy of various compounds on systemic as well as topical applications. Among various drug-carrier systems, poly-lactic glycolic acid (PLGA) matrices have been reported to be nontoxic, biodegradable, and shown to release the entrapped drug gradually over a long duration (**Nair & Laurencin, 2007**).

In the present study, POH-bearing PLGA microparticles were prepared and their efficacy against the skin epidermoid cancer cell line (A253) was evaluated. In addition, potential of POH-bearing PLGA microparticles was evaluated in treatment of di-methyl benzo anthracene (DMBA)-induced tumors in Swiss albino mice.

Materials and methods

All reagents used in the study were of highest purity available. PLGA, poly vinyl alcohol (PVA), and POH were purchased from Sigma Chemical Company (St Louis, MO). Dichloromethane (DCM) was of analytical grade of purity and procured locally. Anti-p53 mutant, anti-p53 wild-type (wt), anti-p21/waf1, anti-tubulin and anti-β-actin antibodies were purchased from BD Biosciences (San Diego, CA).

Preparation of POH-loaded PLGA microparticles

Microparticles used in the present study were prepared by oil-in-water-based emulsion solvent evaporation technique using the published protocol as standardized in our laboratory (**Jeffery *et al.* 1993, Farazuddin *et al.* 2009**). Briefly, a known quantity of POH (30 mg dissolved in minimum volume of methanol), mixed with PLGA solution (190 mg PLGA dissolved in 1.0 mL DCM), and sonicated in a bath-type sonicator to form the primary emulsion. The primary emulsion was mixed with 100 mL of 10% PVA (w/v) and homogenized using a Silverson L4RT homogenizer (Silverson Machines, East Longmeadow, MA). The resulting oil-in-water emulsion was stirred at 25°C for 18 hours to allow solvent evaporation and formation of POH-entrapped microparticles. The microparticles were centrifuged at 10,000 x g for 10 minutes and thoroughly washed with phosphate buffered saline (PBS) (0.15 M NaCl containing 20 mM sodium phosphate, pH 7.4) to remove surface adsorbed drug. The microformulation was lyophilized and finally stored at 4°C until further use.

Entrapment efficiency of POH in microparticles

Entrapment of POH in microparticles was assessed by dissolving an aliquot of the microparticles in 0.1 N NaOH followed by analysis of POH content by high-performance liquid chromatography (HPLC) following the published procedure (**Tao & Pereira, 1998**). Briefly, 10 mg freeze-dried microparticles were dissolved in 1.0 mL of 0.1 N NaOH. The solution was vortexed for 10 minutes followed by centrifugation for 5 minutes at 9168 × *g* at 25°C. An aliquot (100 µL) of supernatant was mixed with 900 µL methanol. The suitable aliquots, of the resultant homogenate solution, were analyzed by reversed phase HPLC using a Symmetry® C-18 column (3.9 mm × 150 mm). The solvent system used was isocratic methanol-water (72:28, v/v). Entrapment of POH was calculated with the help of calibration curve using the pure drug plotted at 220 nm. The percentage entrapment efficiency (% EE) was calculated with the following formula.

% EE = (amount of POH entrapped)/(total amount of POH used in the beginning) × 100.

Scanning electron microscopy of the microparticles

Scanning electron microscopy (SEM) was performed to characterize the size and surface morphology of POH-loaded microparticles using scanning electron microscope (Zeiss EVO 40; Carl Zeiss SMT AG, Oberkochen, Germany). The lyophilized preparation of POH-loaded microparticles was suspended in 20 mM PBS pH 7.4, and a drop of the formulation was mounted on clear glass stub, air dried, and coated with gold-palladium alloy using a sputter coater. An accelerating voltage of 20.00 kV was used for imaging.

Determination of ζ-potential

ζ-potential of the PLGA microparticles was determined using DTS software (Malvern Instruments Ltd, Worcestershire, UK) based on M3-PALS technology. The formulation was lyophilized in a 2.0 mL microfuge tube, and the samples were reconstituted in 20 mM phosphate buffer, pH 7.4. This dispersion was then rapidly dispensed to a electrophoresis cell to measure the electrophoretic mobility, and ζ-potential values were calculated. The experiment was repeated three times, and the average ζ-potential with standard deviation was calculated.

In vitro release kinetics of active POH from PLGA microparticles

To assess the release kinetics of POH from PLGA microparticles, multiple weighed aliquots of the microparticles were dispensed in separate microvials. To each vial, 1.0 mL of 20 mM sterile PBS was added, followed by incubation at 37°C. Aliquots (100 μL) of supernatant were removed after centrifugation at 9168 × *g* for 10 minutes and analyzed for the POH content.

Toxicity tests for POH-bearing PLGA microformulation

A new formulation of a given drug molecule has to be tested for any inherent toxicity before being examined for its efficacy. To settle this issue, toxicity of the in-house prepared formulation was tested both in vitro and in vivo. Preliminary acute drug toxicity was based on in vitro erythrocyte lysis test, wherein hemoglobin, released as a result of membrane leakage or disruption caused by exposure to low doses of the drug, is

measured (**Khan *et al.* 2002**). Briefly, fresh blood was obtained from a healthy rabbit and collected in anticoagulant solution (ethylenediaminetetraacetic acid), followed by centrifugation at 1000 × *g* for 10 minutes at 4°C. Buffy coat as well as plasma was discarded. The washed erythrocytes were diluted with isotonic buffer (20 mM PBS), and 50% hematocrit was prepared. To study the extent of hemolysis, the suspension of red blood cells (RBCs) was incubated with 1.0 mL of free form as well as POH encapsulated in PLGA microparticles (10 mg/mL) at 37°C for 1 hour. Free POH was dissolved in 50 μL of dimethyl sulfoxide (DMSO), and finally volume was made up to 1.0 mL with PBS (final 5% DMSO). After 1 hour, the reaction mixture was centrifuged at 1500 × *g*, and supernatant was collected and analyzed by ultraviolet-visible spectroscopy (λmax = 576 nm) for released hemoglobin. The percentage hemolysis was determined by the following equation:

$$[(\text{AbsT} - \text{AbsC})/\text{Abs}100\% - \text{AbsC})] \times 100,$$

where AbsT is the absorbance of the supernatant from samples incubated with the drugs, AbsC is the absorbance of the supernatant from controls (PBS), and Abs100% is the absorbance of the supernatant of controls incubated in the presence of 1% Triton® X-100, which causes complete lysis of RBCs (total hemolysis).

Hepatic and renal toxicities were monitored by applying multidose regimen (total seven doses, at alternate days) to determine biochemical profiles of serum creatinine and alkaline phosphatase (ALP). The blood was collected by retro-orbital puncture from the mice of different groups after the last administered dosage. The blood was allowed to clot at room temperature, and serum was separated for investigation of creatinine and ALP as per respective guide provided by the manufacturer.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

Epidermoid carcinoma cell line A253 (ATCC HTB-41™) was purchased from ATCC (Manassas, VA) and maintained in Roswell Park Memorial Institute (RPMI) 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum. To perform MTT assay, 5 × 10^4 cells were transferred to each well of a 96-well plate. The plate was incubated for 24 hours, and then increasing concentrations (0-60 μg/mL) of POH were added to each well, three wells received medium only with no POH and served as

control. The plate was incubated for 72 hours, and cell proliferation was measured by adding 20 μL MTT dye (5 mg/mL in PBS) per well. After further incubation for another 4 hours at 37°C in a humidified chamber with 5% CO2, the formazan crystals formed due to reduction of dye by viable cells in each well were dissolved in 150 μL DMSO, and optical density (OD) was read at 620 nm in a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA).

Effect of POH-PLGA-microparticles in induction of apoptosis in the human epidermoid cancer cell line

To examine the in vitro inhibitory effect of POH-bearing PLGA microparticles, the microformulation as well as free form drug was incubated with A253 cells (1×10^8 cells) for 12 and 24 hours. After incubation, the cells were scraped, centrifuged, and washed with RPMI medium and lysed with TNN lysis buffer. The lysate was subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and gels were electroblotted on polyvinylidine fluoride (PVDF) membrane following published protocol (Towbin *et al.* 1979).

Animals

Female Swiss albino mice of weight 20±2 g were obtained from the institute s animal house facility. The animals were housed in poly propylene cages on wood powder bedding in an air-conditioned ambience. Animals were quarantined on equal light/dark cycles (12/12 hour) and were kept on a pellet diet (Ashirwad, Chandigarh, India) and water *ad libitum*. Animals were examined for their mortality and morbidity prior to commencement of the study, and only healthy animals were included in the experiments. The techniques used for administration of various formulations as well as sacrifice of the animals were strictly performed following mandates approved by the animal ethics committee (Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India).

Treatments

Animals in the resting phase of hair cycle were used in the study. The interscapular regions (over an area of 2 cm^2) of the experimental animals were shaven using non

lubricated electric clippers. The mouse skin tumors were induced by using DMBA as carcinogen following the procedure standardized in our laboratory (**Khan *et al.* 2007**). The skin of the shaven dorsal portion of the mice was exposed to DMBA (52 μg in 200 μL acetone) that was applied topically three times a week for 12 weeks. Animals were routinely examined for gross morphological changes on skin and development of tumors. It has been reported that POH at 10 mM concentration does not cause toxicity in a mice model (**Barthelman *et al.* 1997**). This fact was kept in mind when selecting specific dosage regimens of various cream-based POH formulations. The formulations of POH were applied daily with cream base for a period of 7 days after induction of papilloma, with a dose of 10 mM POH in 500 μL cream base per animal. Animals were observed for 1 month to examine the effect of various formulations. The animals were divided into the following groups; each consisting of 15 animals:

Group I untreated control (DMBA applied, followed by treatment with cream base only);
Group II sham PLGA microparticles;
Group III control (healthy animals);
Group IV POH (free form);
Group V POH-bearing PLGA microparticles.

Tumor measurement

Tumor measurement was carried out after 1 month of last dosage application of various POH-based formulations. The diameters of the tumors were measured using a Vernier Caliper, and the tumor volume was determined using the following formula:

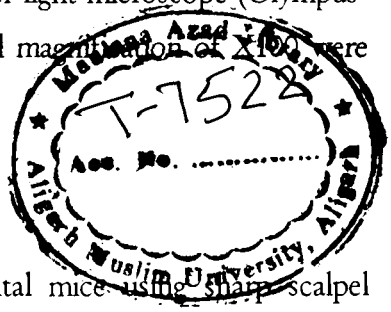
$$V = D \times d2 \times \pi/6,$$

where V = tumor volume, D = biggest dimension, and d = smallest dimension.

Histopathological studies

Animals were sacrificed and their excised papillomas were immersion fixed in 10% formalin. Next, the tissue blocks of 3 × 6 × 5 mm^3 dimensions were processed for paraffin embedding. Thick sections (10 micrometer) were cut with rotary microtome and

stained with H and E stain. Observations were made under light microscope (Olympus-BX 40-Japan), representative photomicrographs with final magnification of X100 were used for comparative study.



Preparation of whole cell fraction

The skin/tumor tissues were removed from experimental mice using sharp scalpel blades. The tissue samples were placed on ice, and fat was scraped off before further processing. The samples were homogenized in the presence of protease inhibitor cocktail, and the whole cell fraction was prepared according to the method published elsewhere (**Serpi** *et al.* 1999).

Western blotting

The tissue homogenate was analyzed for the presence of various apoptotic factors by Western blotting (**Towbin** *et al.* 1979). Briefly, the protein content of the homogenate was determined by Lowry et al method using bovine serum albumin as a standard (**Kocbek** *et al.* 2007). The homogenate (30 μg/well) was subjected to 10%SDS-PAGE under denaturing conditions. The gels were electroblotted onto PVDF membranes, blocked overnight with 5% non-fat dry milk and probed with appropriate antibodies at the dilutions recommended by the suppliers. To quantify equal loading, membranes were re-probed with α-tubulin antibody to determine housekeeping protein tubulin.

Statistical analysis

One-way analysis of variance was used for comparing the mean values of tumor volume between various groups after ascertaining the homogeneity of variance between treatments. Post-hoc analysis for comparing the two groups was done using the least statistical difference technique. The Kaplan-Meier analysis was used to determine survival of tumor-free animals, and differences among various experimental groups were analyzed by log-rank test.

Results

Entrapment efficiency, size, and ζ-potential of PLGA microparticles

Various PLGA-based microparticle formulations have been widely used for the delivery of antibiotics as well as anticancer drugs to accomplish sustained release of the entrapped drugs (**Tao & Pereira 1998, Kocbek *et al.* 2007**). The in-house developed PLGA microparticles had entrapment efficiency of 42.4±3.5, and size was 768±215 nm, as revealed by SEM and Nanophox (Sympatec GmbH, Clausthal-Zellerfeld, Germany) size analyzer (**Figure 2.2A and B**). As ζ-potential acquired by a small sized particle regulates its half-life in vitro as well as in vivo, it was considered important to determine ζ-potential of the in-house prepared microparticles. The POH-PLGA microparticles used in the present study had a ζ-potential of −7.6±0.8 mV.

In vitro release kinetics of POH-loaded PLGA microparticles

In vitro release kinetics of POH-loaded PLGA microparticles was studied at 37°C in PBS, pH 7.4. POH-PLGA microparticles showed sustained release, with only 12% of the entrapped drug leaking out in the initial 18 hours. In the next 12 hours, around 17% of the total drug was released. PLGA microparticles showed an initial burst release pattern followed by sustained release kinetics for extended time period. In the present study, PLGA microparticles were found releasing 22% in the initial 72 hours with an overall total 30% release in 168 hours (**Figure 2.2C**).

In vitro and *in vivo* toxicity of POH-based PLGA microparticle formulation

Before determining the efficacy of any formulation, it is desirable to assess its intrinsic toxicity. With this aim, POH bearing PLGA microparticle (in-house prepared) toxicity was evaluated, and it was found that POH-bearing PLGA microparticles had induced relatively less percent RBC lysis than free form of the drug (**Figure 2.3**). Sham PLGA also induced very negligible lysis.

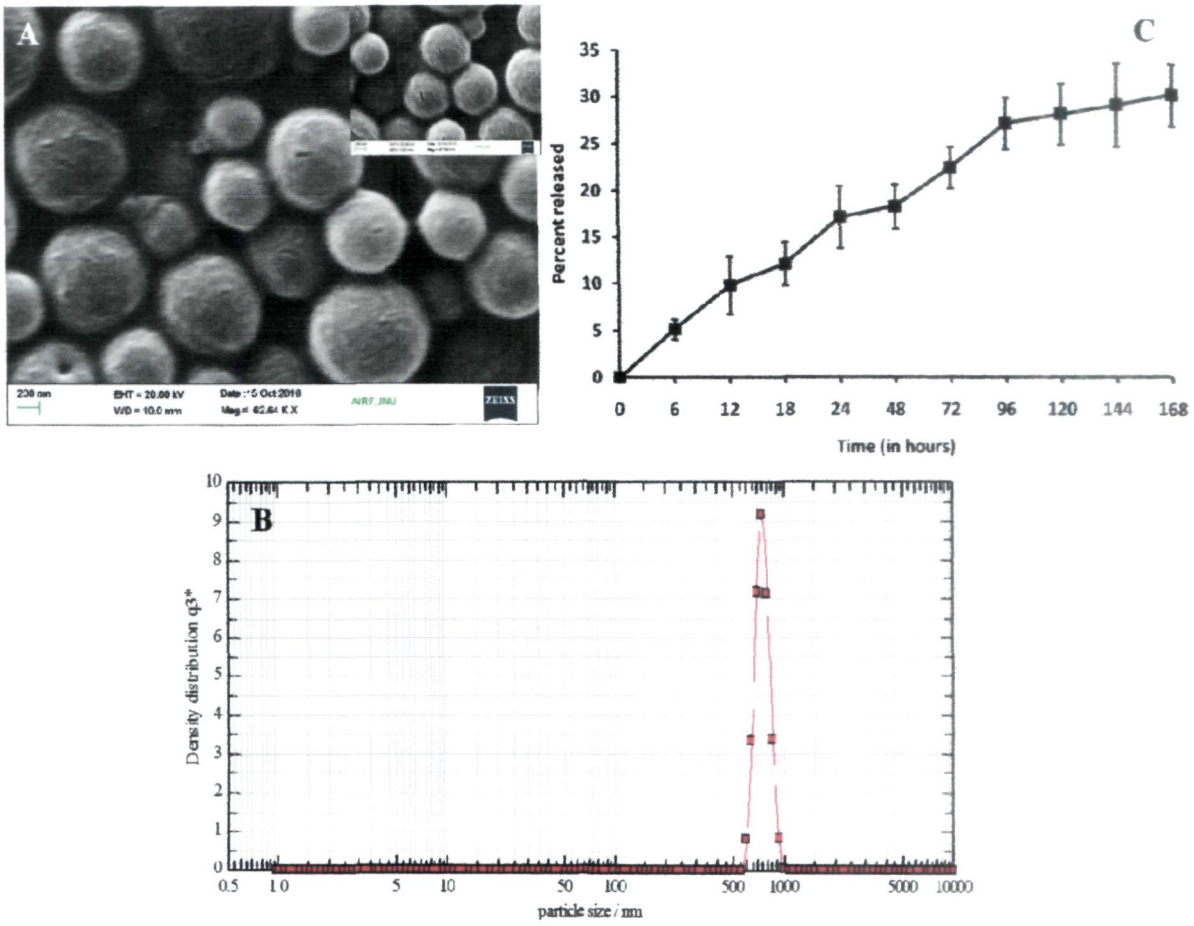


Figure 2.2 (A) Scanning electron microscopy image of POH-loaded PLGA microparticles. **(B)** Corresponding particle analyzer data as obtained by **Nanophox particle size analyzer. (C) In vitro release kinetics of POH from PLGA microparticles.** Release kinetics of POH-PLGA microparticle formulation demonstrates ~30% release of POH from the copolymer at 168 hours, when formulation was dispersed in 20 mM phosphate buffer, pH 7.4 at 37°C. Data are means ± standard deviations of three independent experimental values.

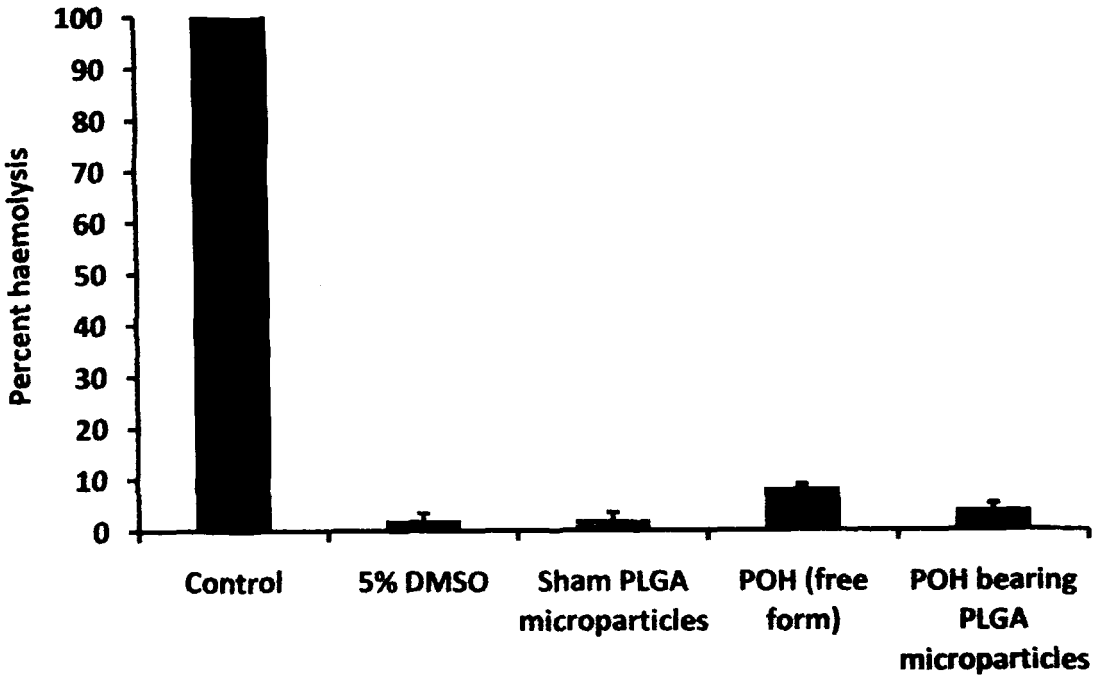


Figure 2.3 Erythrocyte lysis test: *In vitro* toxicity was measured by erythrocyte lysis caused by different POH formulations. Haemolysis test was performed as described in the Materials and methods section. Data represented here are means of three different experiments ± standard deviations.

In another set of experiments, animals treated with multiple dosages of POH-bearing PLGA microparticles were analyzed for liver as well as renal function test parameters to evaluate in vivo toxicity. As shown in **Table 2.1**, POH-bearing PLGA microparticles had comparatively low levels of ALP and creatinine than the free form of the drug. The results established that POH-bearing PLGA microparticles did not have any in vitro and in vivo toxicity and are safe to use.

Table 2.1 Concentrations of creatinine and ALP in plasma of animals treated with POH-bearing PLGA microparticle formulation

Groups	Creatinine (mg %)	ALP (IU/L)
Control	0.32 ± 0.052	32.46 ± 2.12
Sham PLGA microparticles	0.28 ± 0.034	36.88 ± 3.41
POH (free form)	0.35 ± 0.038	44.28 ± 2.32
POH-PLGA microparticles	0.31 ± 0.011	34.25 ± 2.14

Cytotoxic effect of POH-bearing PLGA microparticles

The cytotoxic effect of POH formulations against epidermoid cancer cells was studied using MTT assay as shown in **Figure 2.4**. The data shows that the cytotoxic effect of POH was significant on epidermoid cancer cells at a concentration of 25 μg/mL and resulted in killing of about 50% of the cell population. Nanoparticles are endocytosed by cells faster (**Kocbek *et al.* 2007**), enabling high payload of drug molecules, and exhibit more cytotoxic effect. With this hypothesis, the differential cytotoxicity of free and PLGA microparticle-encapsulated POH at its half maximal inhibitory concentration (IC50) was investigated. As shown in **Figure 2.5**, free POH caused 50% cell death, whereas it increased to ~65% in the POH-PLGA microparticle-treated group in 48 hours incubation (POH-PLGA-microparticle *versus* free POH; $p<0.001$).

In addition, the time-dependent efficacy of POH-bearing microparticles on epidermoid cancer cell system was examined. As shown in **Figure 2.6A**, POH-PLGA microparticles enhanced the expression of p21/waf1 and bax at 12 hours post-incubation, whereas POH in free form was not very effective. Similarly, 24 hours post-incubation, the expression of p21/waf1 in cells treated with POH-PLGA microparticles increased further, while the free form drug was not found to be effective (**Figure 2.6B**). The results clearly suggest that microparticle encapsulated POH is delivered efficiently to the cancer cells and can easily modulate various apoptotic factors and eventually results in apoptosis of the cancer cells.

Effect of POH-PLGA microparticles on regression of tumors and survival of animals

After establishing anticancer efficacy of inhouse-prepared microparticles against cancer cells in vitro, the present study was extended *in vivo* using a mouse model of skin carcinoma. For this purpose, regression in the volume of DMBA-induced tumors was measured after treatment with various POH formulations. As shown in **Figure 2.7**, the percent regression was much higher in the POH-PLGA microparticle-treated group as compared with those receiving the free form of the drug (POH-PLGA-microparticle *versus* free POH $p<0.001$). The treatment with microparticle-based POH formulation resulted in 80% tumor regression, while the free form of drug was able to regress tumor by 65% only. The sham microparticles were devoid of any significant anticancer activity.

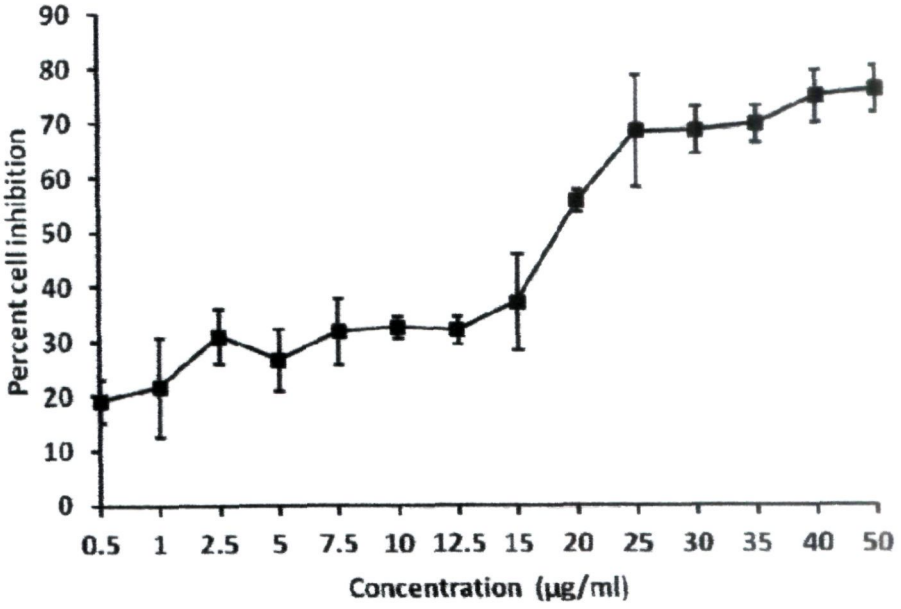


Figure 2.4 In vitro cytotoxicity of perillyl alcohol against A253 cell line as revealed by MTT assay. MTT assay was performed as described in the Materials and methods section. Data represented here are means of three different experiments ± standard deviations.

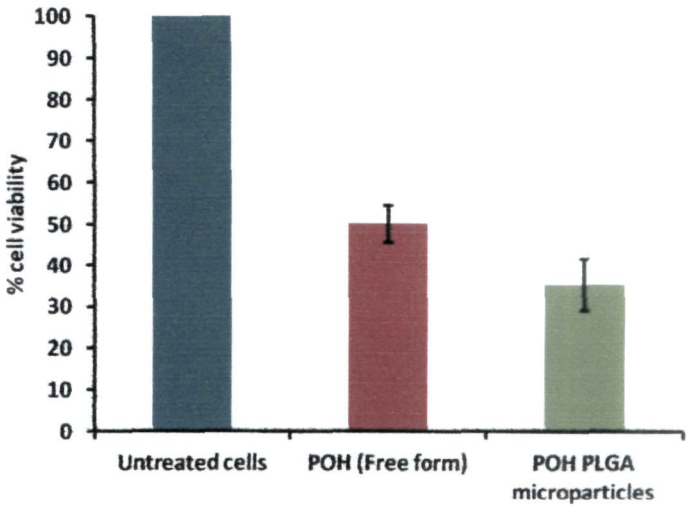


Figure 2.5 Determination of differential cytotoxicity of various formulations of POH using MTT assay. Cells were incubated with various formulations of POH for 48 hours. The percentage cell viability was measured with MTT assay as described in the Materials and methods section. Data represented here are means of three different experiments ± standard deviations (POH-PLGA microparticle *versus* free form POH $p<0.001$).

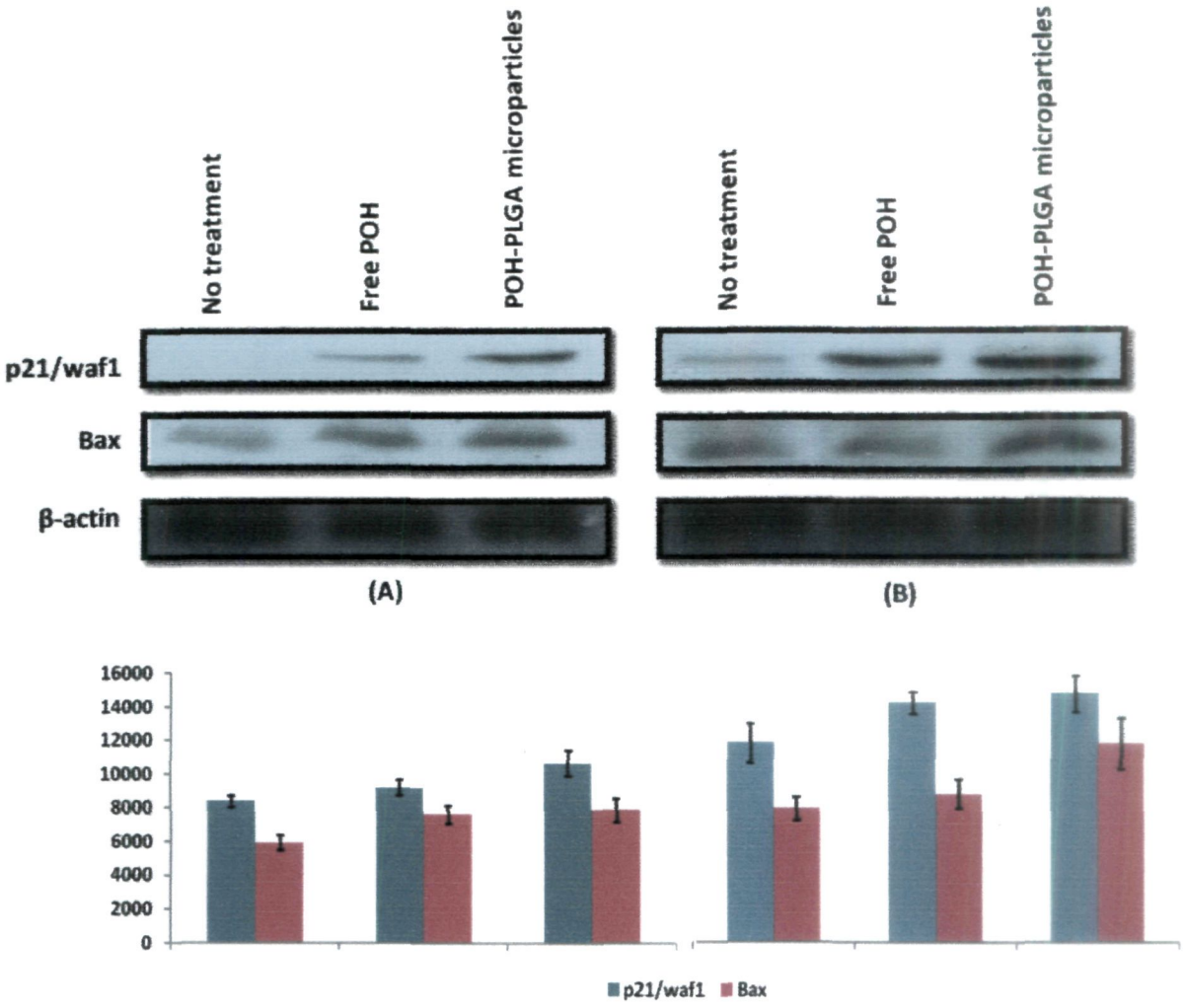


Figure 2.6 Effect of POH-bearing formulations on expression of pro-apoptotic molecules. A253 cancer cells were treated with POH-bearing microformulations for different time periods, and cell lysates were used to examine the expression of apoptotic molecules. **(A)** Expression profile of apoptotic factors at 12 hours post-incubation. **(B)** Expression profile of apoptotic factors at 24 hours post-incubation.

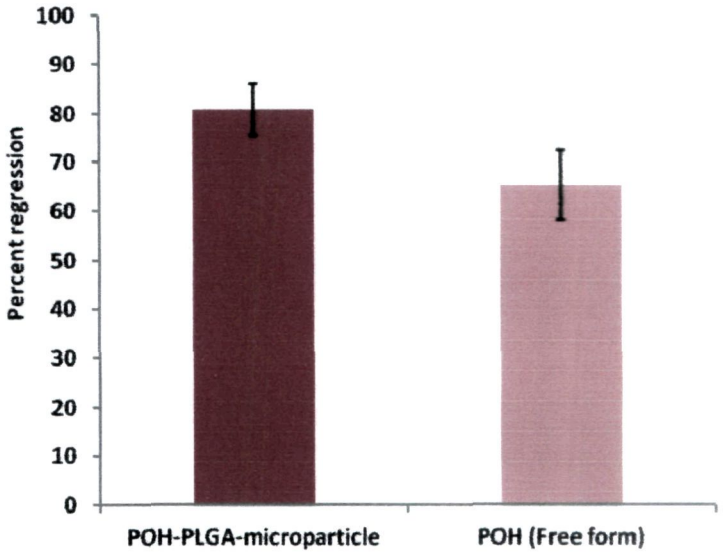


Figure 2.7 Chemotherapeutic effect of two different formulations of POH in regression of tumors in treated animals. Percentage regression was calculated to analyze the most effective formulation of POH. It was assessed by measuring the size with Vernier calipers, and tumor volume was calculated as given in the Materials and methods section (POH PLGA microparticles *versus* free form POH; $p<0.001$). Sham-PLGA microparticles behaved as control.

Next, the efficacy of POH-PLGA microparticles was assessed in terms of the survival of treated animals. Survival graph shows the augmentation of anticancer efficacy of PLGA-encapsulated POH microparticles as well as free form POH against DMBA-induced tumorogenesis at different time points. POH-encapsulated PLGA microparticles showed 80% survival, whereas the group receiving free form of POH resulted in only 40% survival in 13 weeks (POH-PLGA microparticles *versus* free POH; $p<0.05$). None of the animals survived beyond 12 weeks in the control group that was treated with cream base only ($p<0.01$) **(Figure 2.8)**.

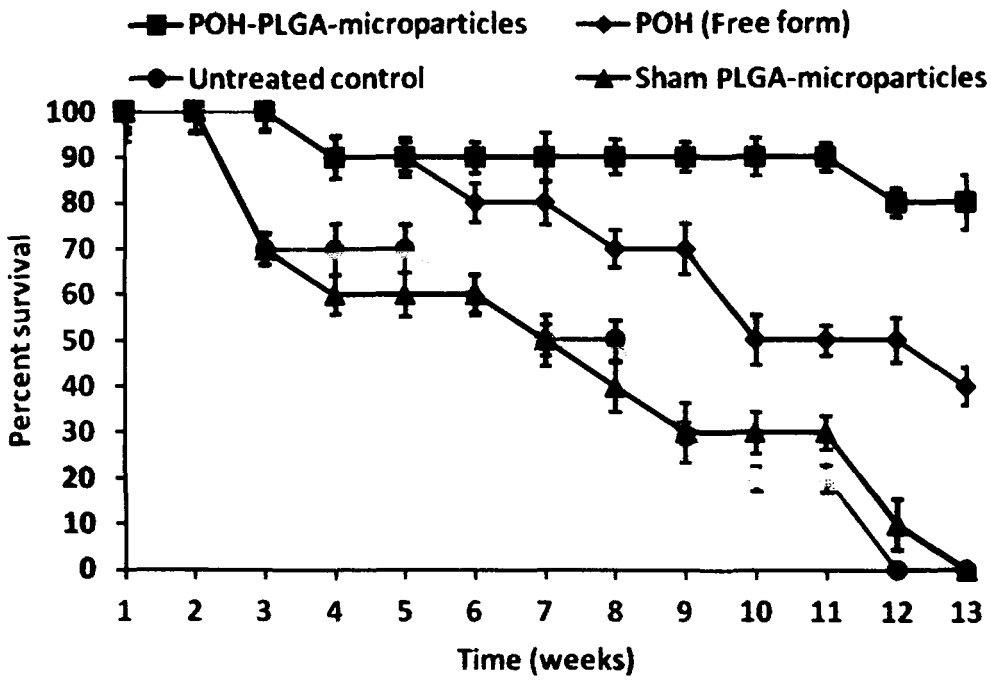


Figure 2.8 Effect of POH-bearing PLGA formulations on survival of DMBA-induced skin papilloma-carrying animals. Kaplan-Meier graph shows the efficacy of various POH formulations in terms of percentage survival after treatment at different time intervals. The study was continued for a period of 13 weeks as described in the Materials and methods section (POH-PLGA microparticles *versus* free form POH; $p<0.05$).

Histopathological analysis

For histopathological studies, skin tissue samples were isolated from animals of various groups and analyzed following the protocol described in Materials and methods. Histopathological studies of tumors identified papillomas in various groups. However, the relative frequency of the individual tumor types differed among animals receiving different treatments. In the skin of healthy animals, there were only a few cell layers thick keratinocytes with mild keratin and pilosebaceous units (**Figure 2.9A**), whereas mice treated with DMBA followed by no POH treatment (positive control) had obvious profuse papillomatous growth with complex fibrovascular core, prominent acanthosis and keratin perl (**Figure 2.9B**). On the other hand, in the free POH treated group, a large amount of keratin and marked acanthosis was visible as depicted in **Figure** 2.9C, while hyperkeratosis, only mild acanthosis, and thin but long papillary growth was observed in animals treated with POH-PLGA microparticles (**Figure 2.9D**).

Western blot analysis

DMBA is generated from incomplete combustion of various organic substances. It is cytotoxic, mutagenic, and inflicts carcinogenic effects in humans and animal models (**Guerin 1978, Dipple *et al.* 1984**). DMBA induces skin papillomas in animal models by upregulating p53 mutant in cancer cells. As shown in **Figure 2.10**, POH-PLGA microparticles downregulate p53-mutant expression significantly when compared with free form of POH. Western blot analysis was also performed to assess expression of p53wt and p21/waf1. POH-PLGA microparticles showedsignificant upregulation of p53wt and p21/waf1 expression in comparison with free form POH (POH-PLGA microparticles *versus* free POH; $p<0.05$).

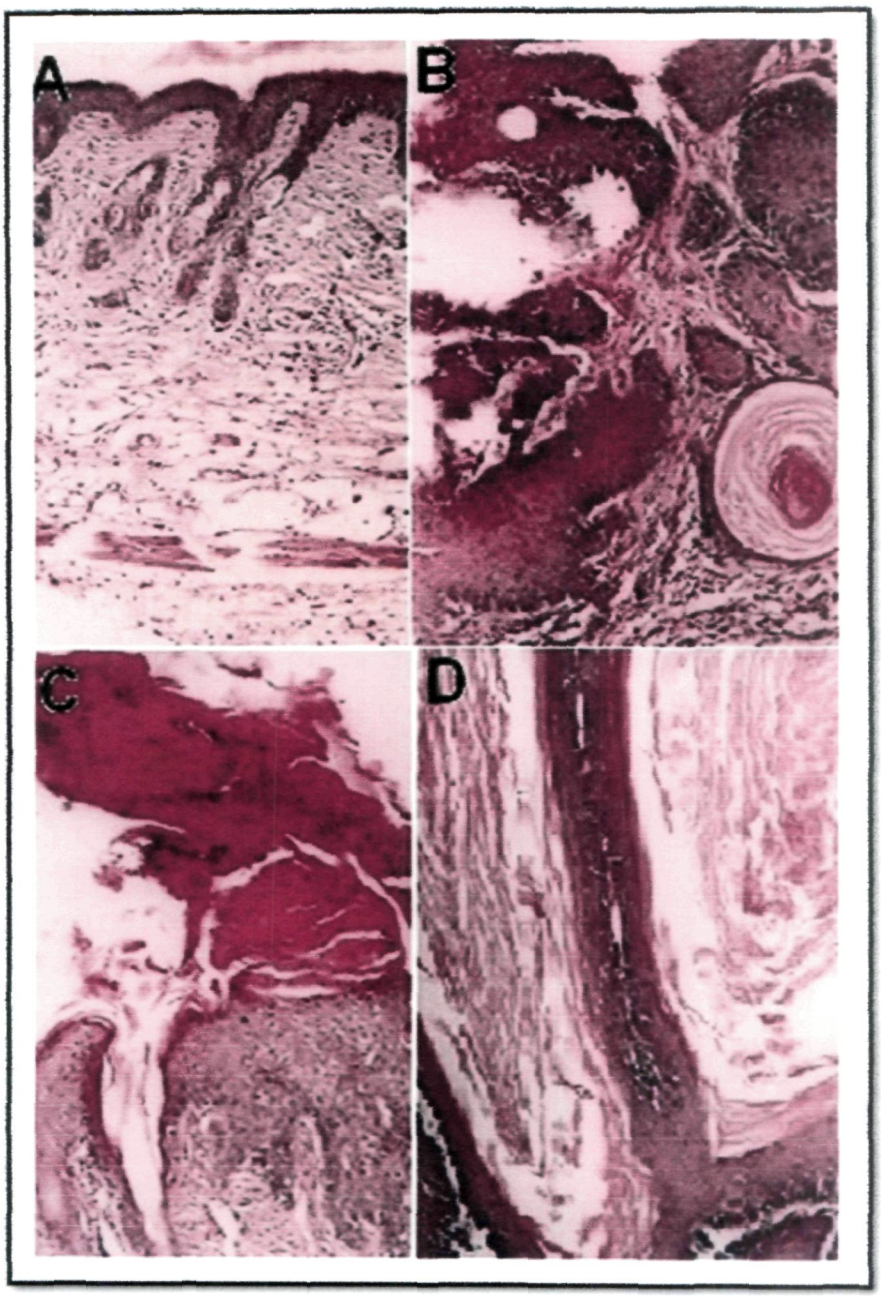


Figure 2.9 Photomicrographs of hematoxylin/eosin-stained sections, showing: (A) normal smooth skin of **healthy animals** with keratinocytes of only a few cell layers thick, mild keratin, and pilosebaceous units; **(B) the positive control** (DMBA treated animals followed by no POH treatment) has profuse papillomatous growth with complex fibrovascular core, prominent acanthosis, and keratin perl; **(C)** treatment with **free form of POH** resulted in large amount of keratin, and marked acanthosis; while **(D)** the treatment with **PLGA microsphere encapsulated POH** resulted in hyperkeratosis, mild acanthosis, and thin but long papillary growth. Magnification 100×.

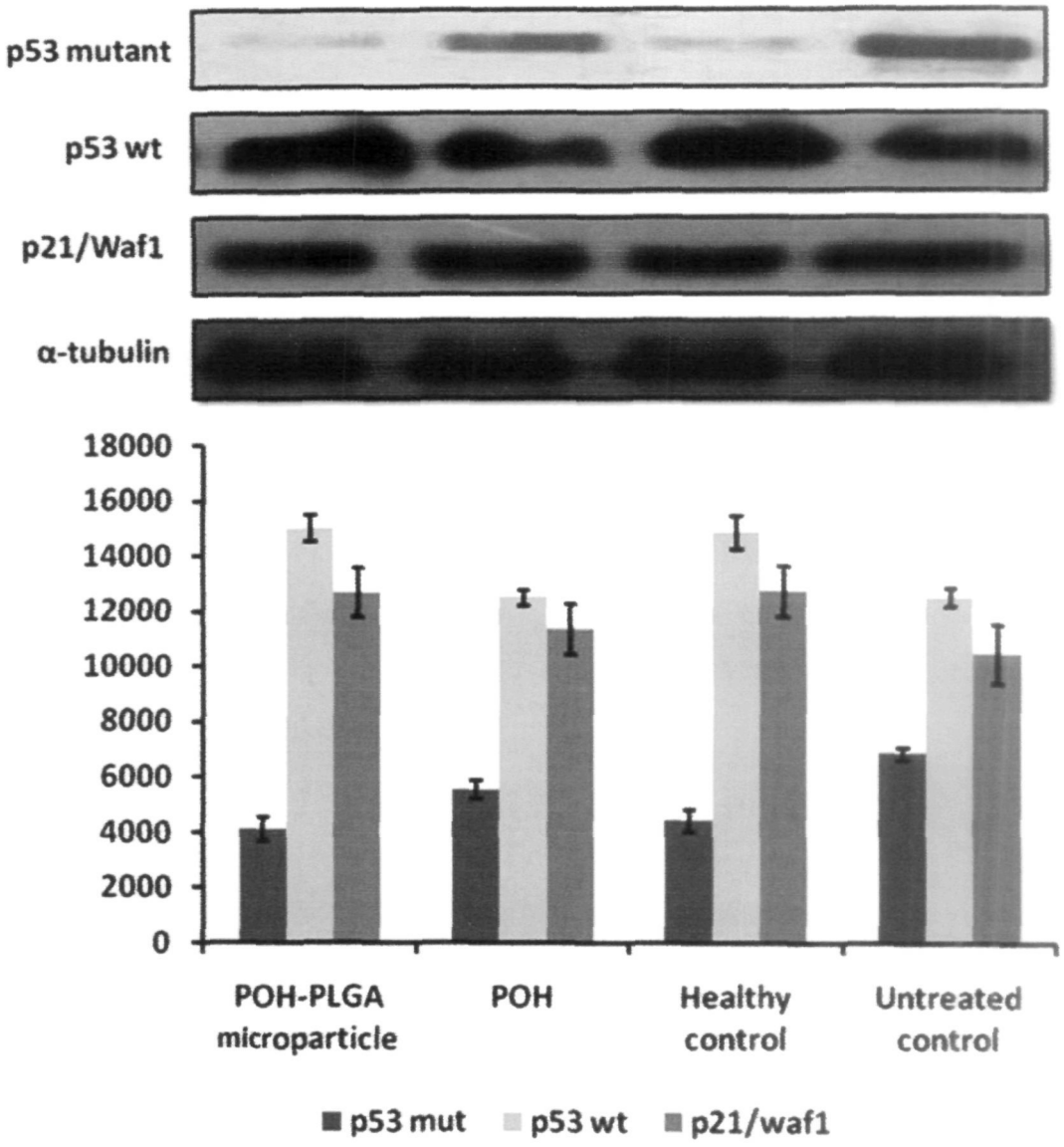


Figure 2.10 Effect of different POH-bearing formulations on the expression of different apoptosis-regulating molecules in treated animals. Skin papilloma cell lysates were prepared as described in the Materials and methods section. Cell lysate was resolved and analyzed using SDS-PAGE. To ensure equal loading, membranes were re-probed for the presence of tubulin, a housekeeping protein, using α-tubulin antibody.

Discussion

Cell-cycle progression in eukaryotes is regulated by several key factors that determine whether the cell will re-enter the cycle, withdraw from it, or undergo differentiation. A slight deviation from the normal course may result in uncontrolled proliferation of the cells that eventually form the basis of cell transformation. In general, malignant state is preceded by several discernible stages, including initiation of DNA damage followed by tumor promotion both in vivo and cancer cells growing in vitro.

A large number of phytochemicals have been reported to prevent or arrest uncontrolled growth of cells through a variety of operative mechanisms. Current global focus is on natural products as a means to control various types of cancers. Some plant-based products possess strong antioxidant properties and have the potential to control or even reverse the process of carcinogenesis and emerge as potential alternatives to chemotherapeutic anticancer agents (**Reddy *et al.* 2003**). However, before developing such potential phytochemicals as prospective therapeutic agents, certain challenges such as palatability, poor solubility, and other related problems have to be addressed. Some of these can be generally circumvented through the use of an appropriate delivery system that has potential to modify the pharmacokinetics of the drug and facilitate its controlled and even preferential release at the desired tumor site.

POH, a dietary constituent and a mono-terpene isolated from cherries, had been used widely as an anticancer agent against various types of cancer in animal models (**Kelloff *et al.* 1996, Broitman *et al.* 1996, Lantry *et al.* 1997**). POH is known to arrest cells in G0/G1 phase and induce apoptosis (**Clark *et al.* 2007**). Although POH is a very effective chemotherapeutic agent, its anticancer properties have not been explored completely. In fact, certain intrinsic properties of POH such as small size, poor solubility in aqueous solution, and bioavailability restrict attainment of its effective concentration at the targeted site and thus limit its application in cancer treatment. Earlier studies suggest that localized delivery at the targeted site could be enhanced significantly by the use of microparticle-based drug delivery and eventually can be successfully employed in tumor therapy (**Vicent & Duncan 2006, Nie *et al.* 2007**). It has recently been demonstrated that naturally occurring phytochemicals can be encapsulated, covalently attached, and adsorbed onto microparticles in order to surmount drug-solubility problems (**Khan *et al.* 2007, Alam *et al.* 2009, Alam *et al.* 2010**). The constituents of the microparticle-based delivery system can also facilitate co-solubilization of the compound/drug in question.

This approach has significant implication in cancer therapeutics because more than nearly half of the phytochemicals showing anticancer activity have solubility constraints (**Merisko-Liversidge *et al.* 2003**). Nanosize range of the microparticles entails a high surface area that not only provides sustained drug release but also provides a useful strategy for their functionalization. The present study focuses on development and characterization of various POH-bearing microparticle formulations and their evaluation in chemotherapy against DMBA-induced skin papilloma in Swiss albino mice.

The POH-bearing PLGA microparticle formulation was characterized for sizing using electron microscopy, and its surface charge properties were assessed on the basis of ζ-potential. The biophysical properties of the novel formulation were also assessed. Further, in vitro anticancer efficacy of PLGA based POH formulation was examined against human epidermoid cancer cell line (A253). Finally, the in vivo therapeutic potential of POH-PLGA microparticle formulations was evaluated on the basis of tumor size regression, survival, histology, and expression profiles of various apoptotic molecules.

To optimize the size and loading efficiency of the active components, a series of PLGA-based microparticles with varying compositions were prepared. The size of the inhouse prepared microparticles was standardized by incorporating PLGA of various molecular-masses. Further, the entrapment efficiency at various added weight percentage values of POH to PLGA was examined. It was found that 50%-70% weight of POH to that of PLGA leads to entrapment efficiency of around 42.4±3.5% with the size of prepared microparticles in the range of 768±215 nm (**Figure 2.2A and B**). This formulation of POH-PLGA was used in subsequent studies.

Usage of the free form drug does not allow attainment of effective concentration unless a large payload is administered, which ironically results in many untoward effects. In such cases, drug-delivery systems offer help by providing continuous and constant supply of the therapeutics for an extended time period. The hydrophobic POH is likely to be physically dispersed by its encapsulation throughout the matrix of PLGA particle. The release kinetics of the entrapped drug showed an overall slow and sustained release that was likely to be regulated by rate of polymer biodegradation. The release kinetics of entrapped drug was determined by incubating the drug-bearing microparticles in PBS, pH 7.4 at 37°C to simulate physiological conditions. The amount of released POH was

estimated by reverse phase HPLC analysis. A linear release of POH accounted for around 22% of the total POH entrapped in PLGA microparticles. In concurrence with earlier reports, where PLGA with the 50:50 ratio of lactic:glycolic acid composition was found to degrade in 1-2 months (**Middleton & Tipton 2000**), the in-house-prepared PLGA microparticles acquired steady release and showed 30% release of entrapped molecules in a time span of 7 days (**Figure 2.2C**).

Both in vitro and in vivo toxicity data revealed that POH bearing PLGA microparticles had negligible toxicity when compared with free form of the drug and thus assures its safety (**Figure 2.3 and Table 2.1**). The cytotoxicity of free POH was next examined, and it was found that POH had an IC50 of 25 μg/mL against A253 human epidermoid cancer cells (**Figure 2.4**). This concentration was used to analyze differential cytotoxicity of free and PLGA microformulation-encapsulated POH and their comparative potential in induction of apoptosis in epidermoid cancer cell line. Due to faster uptake and subsequent release of the drug by POH-PLGA microparticles, it exerts more cytotoxic effect on epidermoid cancer cells than its free form (**Figure 2.5**). This observation could be explained on the premise that application of POH-PLGA microparticles delivers a high amount of active drug at the tumor site mainly. Further, polymer conjugation of low molecular weight drugs alters their biodistribution; enabling their passive targeting and reducing access to sites of toxicity (**Duncan 2006**). Microparticle-based drug formulations entertain a special status in improving the efficacy of drug against many types of cancers. Therefore, the present study was extended to gain insight in POH-PLGA microparticle-mediated modulation of various proteins involved in the apoptosis of A253 cells. Western blot analysis was performed to determine levels of various cell cycle and apoptosis regulating factors. As evident from **Figure 2.6A**, incubation of POH-microparticle formulation with epidermoid cancer cells resulted in enhanced expression of p21/waf1 when compared with free form of POH. The expression of another important pro-apoptotic factor Bax was also analyzed in A253 cells upon their incubation with POH-microparticle formulations. The POH-microparticle formulation up-regulates the expression of Bax, thereby enhancing apoptosis induction in cancer cells (**Figure 2.6A**). In the control groups (cells treated with sham PLGA microparticles), the expression profile of these molecules was not significantly affected when compared with POH-PLGA microparticles, suggesting that microparticle-based formulation is effective in upregulating p21/waf1 and Bax gene expression. The effect of POH-PLGA

microparticles on expression of pro-apoptotic molecules was more prominent after 24 hours incubation (**Figure 2.6B**). Interestingly, the POH-PLGA microparticle was equally successful in downregulating p53-mutant expression in A253 cancer cells (data not shown). Of note, both p53 and/or p21-dependent and -independent pathways have been previously reported to be involved in POH-induced cell cycle arrest and apoptosis in cancer cells; however, the extent of the cell responses varied and can be attributed to the mechanism operative in a specific cell type involved and the doses of the drug used in the study (**Clark *et al.* 2002, Ariazi *et al.* 1999**).

DMBA, a polycyclic aromatic hydrocarbon, is metabolized to chemically reactive electrophile and initiate a cascade of reaction that eventually results in carcinogenesis by covalent interaction(s) with DNA (**Dipple *et al.* 1984**). Further investigations suggest that it induces skin papilloma in animal models by mutagenesis in Ha-ras oncogene (**Bizub *et al.* 1986**).36 Upon topical application of POH-PLGA microparticles, 80.8 ±5.2 regression in tumor size was observed after the treatment, whereas free form POH showed only 65.1 ±7.1 regression (**Figure 2.7**). Increased regression in the POH-PLGA microparticle group could be attributed to sustained release of POH. Interestingly, the tumor regression results were found to have great correlation with survival rate of the treated animals. In case of POH PLGA microparticles, survival rate was highest (80%) over a period of 13 weeks when compared with survival rate of the animals treated with free form POH (40%) (**Figure 2.8**). Further, histopathological analysis of the skin tissues isolated from treated animals was performed and the results clearly demonstrated that free form POH had more acanthosis and papillary growth than POH-bearing PLGA microformulations (**Figure 2.9**). The pro-apoptotic gene p53 regulates the balance between cell proliferation and cell death (**Croce 2008, Knudson 2001**). It has been observed that the p53 gene gets mutated in most types of the malignancies, including sarcomas. Upon exposure to a potent carcinogen, the toxic insult of the cell is generally nullified by a chain of cell cycle regulatory events programmed to check the uncontrolled proliferation by a suitable repair mechanism (**Branzei & Foiani 2008**). The failure to induce expression of functional p53wt leads to deregulation of cell cycle arrest or cell death and tumor progression. The results of the present study showed decreased p53-mutant expression upon treatment with POH-bearing PLGA microparticles. Also, higher efficacy of the microparticle-based formulation of POH can be correlated with upregulated expression of p53wt and p21/waf1 genes (**Figure 2.10**).

The survival data further establish higher supremacy of POH-PLGA microparticles over its free form. The observed higher efficacy of POH-bearing PLGA microparticle formulations can possibly be attributed to the greater bioavailability of POH and its accumulation at the tumor site. Finally, it can be inferred that microparticle-based formulations not only overcome the solubility constraints of the poorly water soluble POH but also facilitate its release in regulated fashion that eventually results in better efficacy of POH in terms of tumor regression and survival of treated animals.

Chapter 3:

Chemotherapeutic efficacy of curcumin bearing microcells

Hepatocellular carcinoma (HCC) is a malignancy of worldwide significance and has become an increasingly important subject in the United States. Currently HCC is the fifth most common solid tumor across the globe and the fourth leading cause of cancer-related death (**Parkin *et al.* 1999**). Treatment modalities for the HCC have been well documented (**Johnson 2000, Curley 2000, Nakakura & Choti 2000**). Despite availability of innumerable options, diagnosis of HCC remains underscored at early stage. Most of the published research work shows that systemic chemotherapy response rates between 0-25%, and in spite of therapy it has never showed prolonged survival in treated individual (**Simonetti *et al.* 1997**). So there is a growing need for new anticancer agents that could provide an alternative to synthetic drugs. In a surge to discover novel chemotherapeutic agents natural products are providing templates.

Recent studies on tumor inhibitory compounds of plant origin have resulted in an array of novel therapeutics. Different phytochemicals including dietary agents and nutrients have been found effective against various types of cancer. Those active substances include vitamin derivatives, flavenoic and phenolic derivatives, sulphur containing compounds and isothiocyanates. One such phytochemical, curcumin (difruloyl methane) found in large quantities in the roots of *Curcuma longa* (**Shishodia *et al.* 2005**), has been used as a spice and colouring agent in Indian and Chinese food for centuries, also it has been utilised as therapeutic agent (**Figure 3.1**). Curcumin possess antioxidant, anti-inflammatory properties and inhibits chemically induced carcinogenesis in the skin, fore-stomach, colon, and liver (**Inano *et al.* 1999, Chuang *et al.* 2000, Singh *et al.* 1998, Li *et al.* 2002, Kawamori *et al.* 1999**).

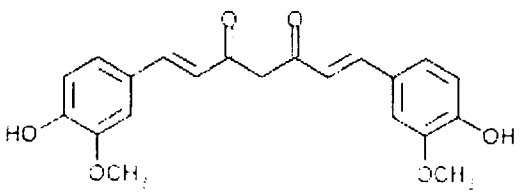


Figure 3.1 Chemical structure of curcumin

Despite their high efficacy, phytochemicals possess high dose associated toxicity and other side effects. Moreover, their non-targeted accumulation, fast elimination and poor solubility complicate the issue. Novel chemotherapeutic agents identified through different combinational screening procedures have solubility problem (**Merisko-**

Liversidge *et al.* 2003). Although curcumin does not possess any toxicity even at high dosage but its bioavailability is very poor. That is the reason why it has not been translated into clinical settings yet. There has been intensive research to overcome its bioavailability and solubility issue. With the aim to increase its bioavailability, we encapsulated it in different types of microparticle based formulations. Choice of polymers to be used for the formation of microparticles should be made according to the application. In case of cancer therapeutics, it should have good entrapment efficiency and longer degradation period to ascertain sustained release. We opted for poly lactic glycolic acid (PLGA) and phosphatidyl choline (PC) to encapsulate curcumin. PLGA is non-enzymatically degradable polymer. Its degradability depends on the molar ratio of lactic:glycolic acid (**Middleton & Tipton 2000**). We used PLGA with molar ratio of lactic:glycolic 50:50, as it has been used widely for drug delivery applications. We also entrapped curcumin bearing PLGA microparticles in PC liposomes (microcells) to resolve burst release issue. Thus formulating such compounds using different delivery systems could be a plausible solution. PLGA and PC have been used extensively to formulate such compounds, also their formulation showed better efficacy and enhanced activity than the native molecule (**Farazuddin *et al.* 2011**). Both of them have advantage of being biodegradable and non-toxic (**Verma *et al.* 2011, Pietzyk & Henschke 2000**) and have been used widely in treatment of infectious diseases and cancer as well (**Jain *et al.* 2011, Riganti *et al.* 2011**).

In the present study, we report synthesis of curcumin bearing microcells and compare their efficacy with PLGA microparticles. We also studied their toxicity and curcumin biodistribution along with its application in treatment of di ethyl nitrosamine (DEN) induced hepatocellular carcinoma.

Materials and methods

Curcumin, egg PC, cholesterol, PLGA and poly vinyl alcohol (PVA) were procured from Sigma Aldrich Company (St. Louis, USA). Anti-p53 wild type, anti-bax, anti-bcl-2 and anti-β-actin antibodies were purchased from BD Biosciences (San Diego, CA). Liver and kidney function tests were performed using the kits, bought from Span diagnostics, India. Rest of the chemicals were of analytical grade and procured locally.

Preparation of Curcumin bearing PLGA microparticles

Microparticles used in the present study were prepared by oil-in-water (O/W) emulsion solvent evaporation technique using published protocol as standardized in our laboratory (**Jeffery *et al.* 1993**). Briefly curcumin (30 mg dissolved in minimum volume of DMSO) was mixed with PLGA solution (190 mg PLGA dissolved in 0.5 mL di chloro methane) and sonicated in bath type sonicator to emulsify. The emulsion was added to 10% PVA solution (50 mL) and homogenized using a Silverson L4RT Homogenizer (Silverson Machines, East Longmeadow, MA) and the resulting suspension was stirred under ambient temperature (25°C) for 18 hours to allow solvent evaporation leading to curcumin loaded microparticle formation. The microparticles were centrifuged at 10,000 x g for 10 minutes and washed with PBS (pH 7.4), to remove unentrapped drug. The microparticle based formulation was lyophilized and finally stored at 4°C till further use.

Preparation of PLGA microparticles bearing microcells

Curcumin bearing microparticles were entrapped in PC liposomes by freeze and thaw method. Briefly PC liposomes were prepared from egg PC (49 μmol) and cholesterol (21 μmol) using published method with some modifications as standardized in our laboratory (**Khan *et al.* 2004**). Curcumin bearing PLGA microparticles were mixed with blank PC liposomes and several cycles of freeze and thaw were repeated for their encapsulation. The smallest fraction of PLGA microparticles was used for encapsulation in PC liposomes. This was achieved by centrifuging prepared PLGA microparticles at 2000 x g for 5 minutes and supernatant having small size microparticles were separated. After freeze and thaw cycles microcells bearing suspension was subjected to sonication for 5 minutes in a water bath sonicator (Power Sonics 405, Seoul, Korea). This suspension was centrifuged further to 5000 x g for 5 minutes. After centrifugation supernatant having blank liposomes was discarded and middle layer having only curcumin loaded

microparticles was separated from microcells. This fraction of microcells was further used for its characterization.

Entrapment efficiency of curcumin in PLGA microparticles and microcells

The entrapment efficiency of curcumin in PLGA microparticles and microcells, was measured by dissolving 10 mg of freeze dried formulations in 1.0 mL of 0.1 N NaOH (**Jeffery *et al.* 1993**). This solution was vortexed and then incubated at 37°C for 1 hour. After incubation solution was centrifuged at 9168 x g at 25°C for 10 minutes and supernatant was examined for curcumin using HPLC (**Jayaprakasha *et al.* 2002**). Briefly, an aliquot (100 μL) of supernatant was mixed with 900 μL methanol. The suitable aliquots, of the resultant homogenate solution, were analyzed by reversed phase HPLC using a Symmetry® C-18 column (3.9 mm x 150 mm). The solvent system used was isocratic acetonitrile:methanol:water (55:35:10 v/v). A standard curve of the drug was plotted at 425 nm by determining the area under curve corresponding to the known (increasing) amount of the drug.

In vitro release kinetics of curcumin from PLGA microparticles and microcells

For PLGA microparticles and microcells, multiple samples of given formulation were weighed and dispensed into various micro vials. To each vial, 1.0 mL of 20 mM sterile PB was added. The vials were placed at 37°C. 100 μl of PB was removed by centrifugation at 9168 x g for 10 minutes and fresh buffer was exchanged at every time point thereafter. The content of curcumin was determined as described previously (**Jayaprakasha *et al.* 2002**). Released curcumin concentration was calculated from the standard curve of the drug plotted in presence of 20 mM PB.

Animals

For *in vivo* studies female Swiss albino mice (weight 20±2g) were obtained from institute's animal house facility and were kept on standard pellet diet (Hindustan lever ltd) and water *ad libitum*. Animals were checked twice daily for mortality and morbidity prior to the commencement of the study and only healthy animals were used for *in vivo* experiments. The techniques used for mice handling, bleeding, injection and sacrifice

were strictly performed as per the guidelines of CPCSEA (committee for the purpose of control and supervision of experiments on animals), Govt. Of India.

Toxicity tests for curcumin formulations

A new formulation of any drug molecule has to be tested for any inherent toxicity before being used for efficacy studies. Toxicity issues were tested both *in vitro* and *in vivo*. Preliminary acute drug toxicity was tested through *in vitro* erythrocyte lysis test wherein haemoglobin, released as a result of membrane leakage or disruption caused by exposure to therapeutic dosages of the drug, is measured (**Khan *et al.* 2002**). Briefly, fresh blood was obtained from a healthy rabbit and collected in anticoagulant solution (EDTA), followed by centrifugation at 9168 x g for 10 minutes at 4°C. Buffy coat as well as plasma was discarded and erythrocytes were washed with PBS, pH 7.4 thrice. The washed erythrocytes were diluted with isotonic buffer (20mM PBS) and 50% haematocrit was prepared. To study extent of haemolysis, the suspension of RBCs was incubated with 1.0 mL of free form as well as microcells and microparticles encapsulated curcumin (10 mg/mL) at 37°C for 1 hour. Free form of curcumin was dissolved in 50 μL of DMSO and finally volume was made up to 1.0 mL with PBS (final 5% DMSO). After 1 hour the reaction mixture was centrifuged at 1500 x g and supernatant was collected and analyzed by UV-Visible spectroscopy (λmax=576nm) for released haemoglobin. The percent haemolysis was determined by the following equation:

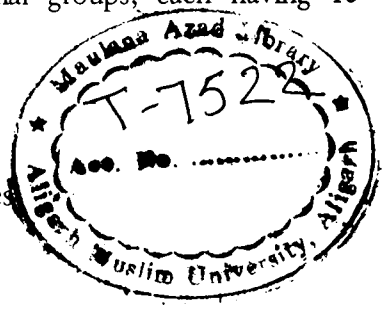
$$[(AbT - AbsC)/Abs100\% - AbsC)] \text{ X } 100$$

where AbsT is the absorbance of the supernatant from samples incubated with the drugs, AbsC is the absorbance of the supernatant from controls (PBS) and Abs100% is the absorbance of the supernatant of controls incubated in the presence of 1% Triton X-100 which causes complete lysis of RBCs (total haemolysis).

Hepatic and renal toxicities were monitored by applying multidose regimen (total three doses, at alternate days) to determine biochemical profiles of serum creatinine and alkaline phosphatase (ALP). The blood was collected by retro-orbital puncture from the mice of different groups after last dosage. The blood was allowed to clot at room temperature and serum was separated for investigation of creatinine and ALP as per respective guide provided by the manufacturer.

In vivo bio-distribution studies of curcumin

In vivo biodistribution studies were carried out in Swiss albino mice weighing 20±2 g. Biodistribution studies had following experimental animal groups, each having 10 animals.



Group I	Free form of curcumin
Group II	Curcumin encapsulated PLGA microparticles
Group III	Curcumin bearing microcells

Different curcumin loaded formulations as well free form of curcumin, were injected via intraperitoneal (*i.p.*) route at a dose of 200 mg/kg b.w. of animal. Only single injection was administered. At different time points blood was collected by retro orbital puncture and animals were sacrificed for the quantification of drug. Similarly, different organs (liver, kidney and spleen) were taken out aseptically, washed with RPMI medium. Organs were rinsed using tissue paper and weighed. Now organs were homogenized using HPLC buffer and centrifuged at 9168 x g. Supernatant was collected for HPLC examination and the amount was calculated from the standard plotted with respective organ. In case of blood, it was centrifuged at 1000 x g for 10 minutes and serum was collected. Serum proteins were precipitated with the HPLC buffer and centrifuged at 9168 x g for 10 minutes. Supernatant was collected and examined for curcumin concentration.

Induction of Liver cancer by di-ethyl nitrosamine

Liver cancer in experimental models was induced as described elsewhere and as standardized in our laboratory (**Pitot *et al.* 1978**). Briefly DEN at a dose of 2.4 mg per animal was injected intraperitonealy (*i.p.*) and monitored for 40 days. After incubation period treatment was started.

Assessment of anticancer efficacy

The efficacy of curcumin based formulations was assessed by examining various parameters including liver enzymes, percent survival, liver histology and Western blot profile of apoptotic molecules. Curcumin was used at a dose of 50 mg/kg body weight as this dose already has been shown to reduce tumor incidence and increased survival in

ascites mice model (**Kuttan *et al.* 1985**). All formulations of curcumin were given through *i.p.* route for a period of 10 days after induction of tumor and then were left for resting period (30 days) to observe the effect of formulations. Animals were divided into following groups. Each experimental group had 10 animals.

Group I	Healthy control
Group II	Untreated control (DEN treated only)
Group III	Sham PLGA microparticles
Group IV	Sham microcells
Group V	Free curcumin (50 mg/kg)
Group VI	Curcumin loaded PLGA microparticles (50 mg/kg)
Group VII	Curcumin bearing microcells (50 mg/kg)

Assessment of Liver enzymes

It had been shown that liver enzymes get up-regulated in cancerous conditions (**Carr *et al.* 2010**). Hence efficacy of curcumin based formulations was assessed by monitoring the level of different liver enzymes *viz*: alkaline phosphatase (ALP), aspartate transaminase (AST) and gamma glutamyl transferase (GGT) in treated animals.

TNF-α expression

TNF-α is a member of TNF superfamily and contributes in immunity, also takes part in apoptosis and cell survival (**Aggarwal *et al.* 2006**). TNF-α concentration was measured from serum samples of various treated groups using ELISA. ELISA was performed as per the manufacturer's guidelines. Briefly, 50 μL of the purified capture antibody was coated on polystyrene microtitre plates at 4°C in carbonate buffer pH 9.4 overnight. After incubation plates were washed five times with PBST and blocked with 5% skimmed milk. After 1 hour incubation at 37°C, plates were washed with PBST and incubated with 50 μL sample for the detection of cytokine. After 2 hours of incubation at 37°C plates were washed and coated with biotinylated anti-mouse TNF- α (detection antibody). Now plates were washed thrice with PBST and 100 μL of streptavidin-HRP was added. It was incubated for 30 minutes at room temperature. Plates were again washed three times with PBST and finally developed with tetra methyl benzidine. The absorbance was read at 450 nm with microtitre plate reader. A standard was also plotted using (known concentration) of recombinant TNF-α.

Preparation of whole cell fraction

The liver was removed from experimental mice and was placed on ice. The samples were homogenized in the presence of protease inhibitor cocktail, and the nuclear fraction was prepared according to the protocol published elsewhere (**Serpi R*et al.* 1999**).

Western Blotting

The while cell fraction was analyzed for the presence of various apoptotic molecules using Western blotting method (**Towbin *et al.* 1979**). Briefly, the protein content of the homogenate was estimated by the routine method using bovine serum albumin as a standard (**Lowry *et al.* 1951**). Proteins (30μg/well) were resolved under denaturing conditions on 10% SDS-PAGE and electro blotted onto nitrocellulose membranes. The blots were blocked overnight with 5% non-fat dry milk and probed with appropriate antibodies at the dilutions recommended by the suppliers. To quantify equal loading, membranes were reported with α-tubulin antibody. Data are presented as the relative pixel density of each band normalized to a band of α-tubulin. The intensity of the band was quantitated using image analysis software an Image gel documentation system.

Statistical analysis

All graphs were plotted using MS-Office excel 2007. Different analyses were done using SigmaPlot 11.0. The Kaplan-Meier analysis was used to estimate survival and differences were analyzed by log-rank test.

Results

Characterization of curcumin bearing PLGA microparticles and microcells

Sizing of the prepared microcells were done using Nanophox size analyzer. In the previous chapter, we reported the size of PLGA microparticles to be 768±215 nm but after centrifuging the aliquot small size microparticles used for encapsulation in PC liposomes were found to be of the size 510±82 nm. In the present study, we observed that upon encapsulation of PLGA microparticles in PC liposomes their size was increased and, in-house prepared microcells had average size of 810±188 nm (**Figure 3.2A**). Further, we performed release kinetics of curcumin from different formulations using PB (pH 7.4) at 37°C. PLGA microparticles showed 17% release of curcumin in 2 days which increased very slowly till 7th day (28%) whereas in case of curcumin bearing microcells release was comparatively very slow. In 2 days, it released 4% content which increased upto 16% in 7 days time span (**Figure 3.2B**). Curcumin bearing PLGA microparticles showed burst release pattern whereas microcells had very sustained release initially that increased gradually over extended time period (**Figure 3.2B**).

Toxicity Test

In vitro toxicity

Newly developed various curcumin based formulations were evaluated for *in vitro* toxicity by RBC lysis test. **Figure 3.3** shows the lysis caused by different curcumin based formulations. 100% lysis of RBC caused by Triton-X-100 was considered as total RBC lysis. Lytic ability of blank microparticles and microcells was also examined. In the **Figure 3.3** it's shown that curcumin bearing PLGA microparticle and microcells caused negligible lysis as compared to free form of curcumin.

In vivo toxicity

In vivo toxicity of different curcumin based formulations was examined by estimation of hepatic and renal parameters. Animals were treated with different curcumin dosages as described in earlier section and serum was isolated for the evaluation of creatinine and ALP. Untreated animals served as control for the comparison study. As showed in **Table 3.1** curcumin based formulations did not have elevated renal and liver function parameters.

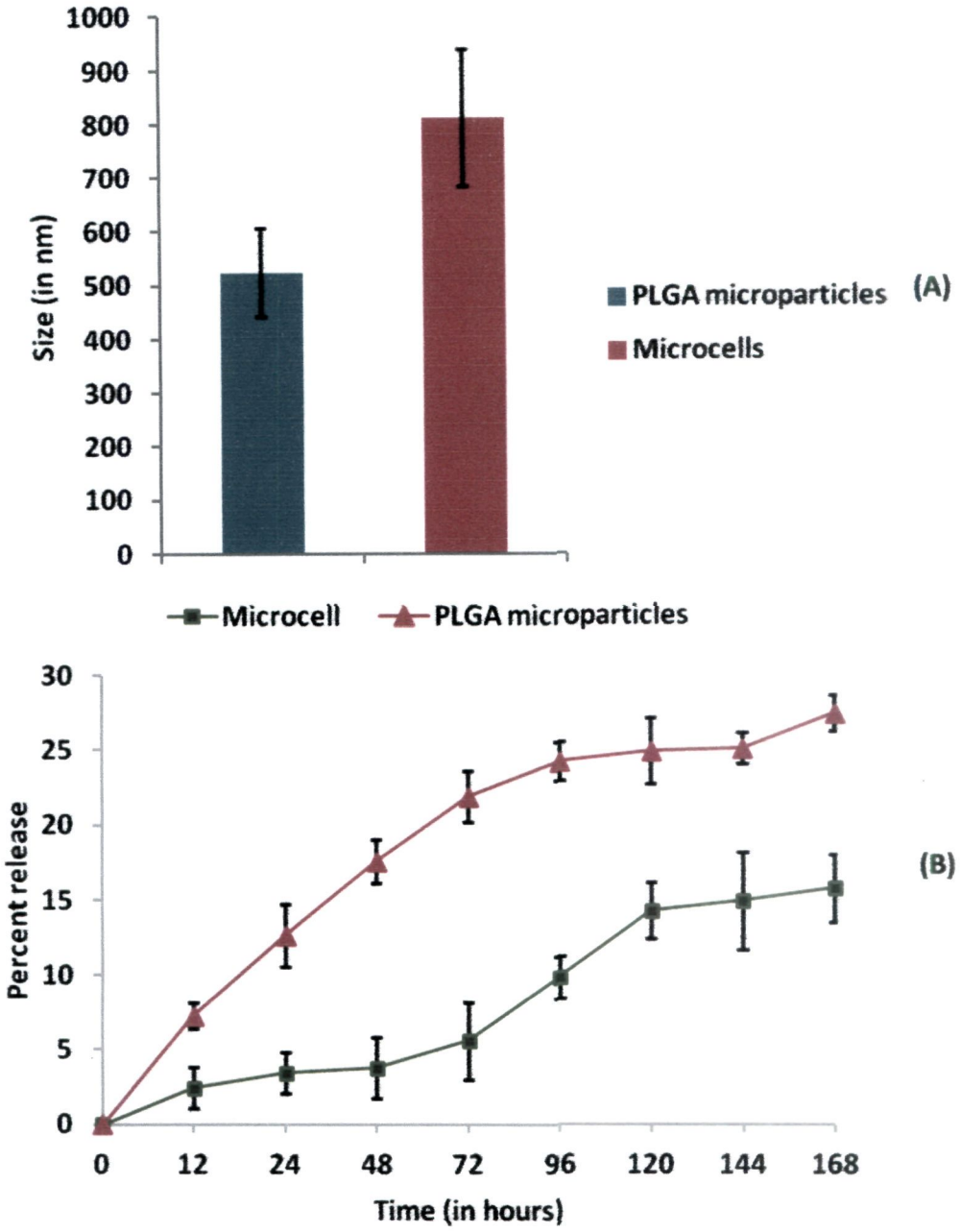


Figure 3.2 Characterization of curcumin bearing microcells. Size characterized using Nanophox size analyzer (A), release kinetics of curcumin from microcell and PLGA microparticles in 20mM PBS (pH 7.4) at 37°C using HPLC (B). Curcumin release kinetics was determined as described in materials and methods. Data represented here is mean of three different experiments ± SD.

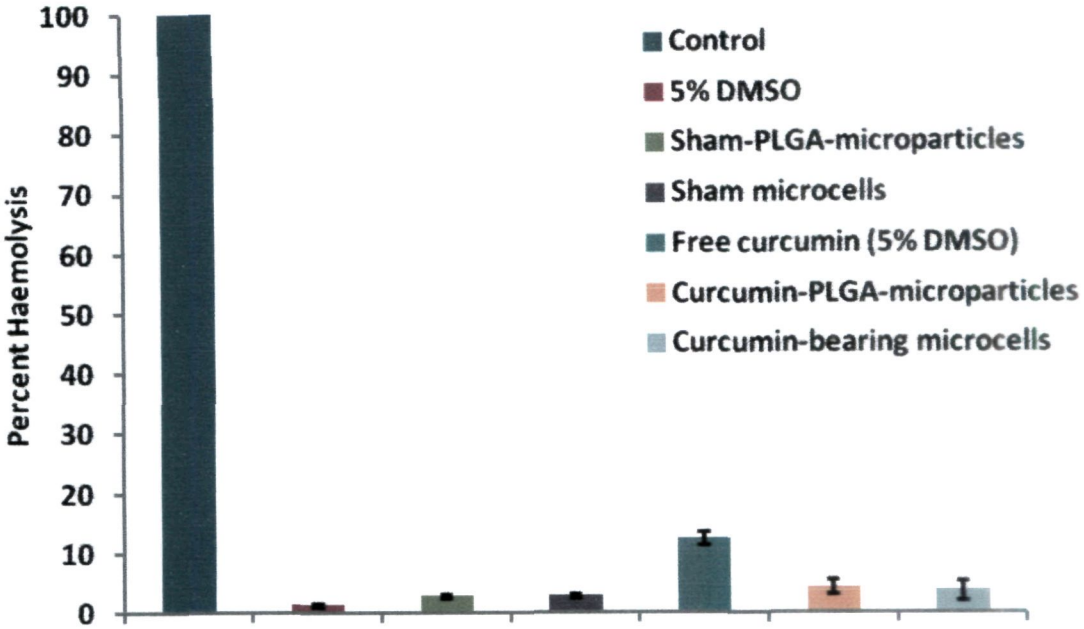


Figure 3.3 Erythrocyte lysis test: *In vitro* toxicity was measured by erythrocyte lysis caused by different curcumin formulations. Haemolysis test was performed as described in materials and methods. Data represented here is mean of three different experiments ± SD.

Table 3.1 Concentrations of creatinine and ALP in plasma of animals treated with curcumin formulations.

Groups	Creatinine (mg %)	ALP (IU/L)
Control	0.27±0.052	32.23±2.10
Sham PLGA microparticles	0.30±0.28	38.42±1.46
Sham microcells	0.24±0.034	33.82±3.40
Free curcumin (50 mg/kg)	0.36±0.038	42.39±1.29
Curcumin PLGA microparticles (50 mg/kg)	0.28±0.32	35.12±2.42
Curcumin bearing microcells (50 mg/kg)	0.22±0.011	36.65±2.28

Bio-distribution of curcumin

Numerous reports have been published about the curcumin formulation development and their *in vitro* efficacy but they do lack data of its bio-distribution (**Karikar *et al.* 2007, Li *et al.* 2005**). We developed curcumin based PLGA microparticles and encapsulated them in PC liposomes with the aim to increase its bioavailability and in turn to enhance its shelf life and *in vivo* anti-cancer efficacy.

As shown in **Figure 3.4A,** liver tissue had very high concentration of curcumin at 2 hour post injection which declined very fast in the free form treated group. After 24 hours, it was not detectable. In case of PLGA microparticles treated animals, initially lesser amount of curcumin was detected which increased upto 12 hours post injection. After that its concentration reduced but it was still detectable 24 hours post injection. We observed increasing concentration of curcumin in microcells treated animals. Initially it had 29.65 μg/g concentration which increased upto 78.8 μg/g after 72 hours post injection.

Further curcumin's bio-distribution was examined in kidneys of treated animals. As shown in **Figure 3.4B,** free form treated group had 29.5 μg/g curcumin at 2 hours post injection. Its concentration was decreased further and only 5.21 μg/g curcumin was detected after 24 hours. PLGA microparticles treated animals had 32.61 μg/g initially which was decreased further but the concentration was still detectable after 72 hours (0.38 μg/g). Microcells treated group showed increasing pattern of the curcumin and at starting point it had 7.08 μg/g that increased upto 28.42 μg/g at 48 hours.

We also examined serum curcumin concentration in different curcumin formulation treated groups. In free curcumin treated group, curcumin level was very high at 2 hours post injection that came down very fast and could not be detected beyond 24 hours post treatment. Although PLGA microparticles and microcells did not have high concentrations of the compound initially, but it increased slowly and both groups had highest curcumin concentrations 13.27 and 19.99 μg/g at 24 hours. Beyond this time point curcumin level declined in both the treated groups although it was still detectable.

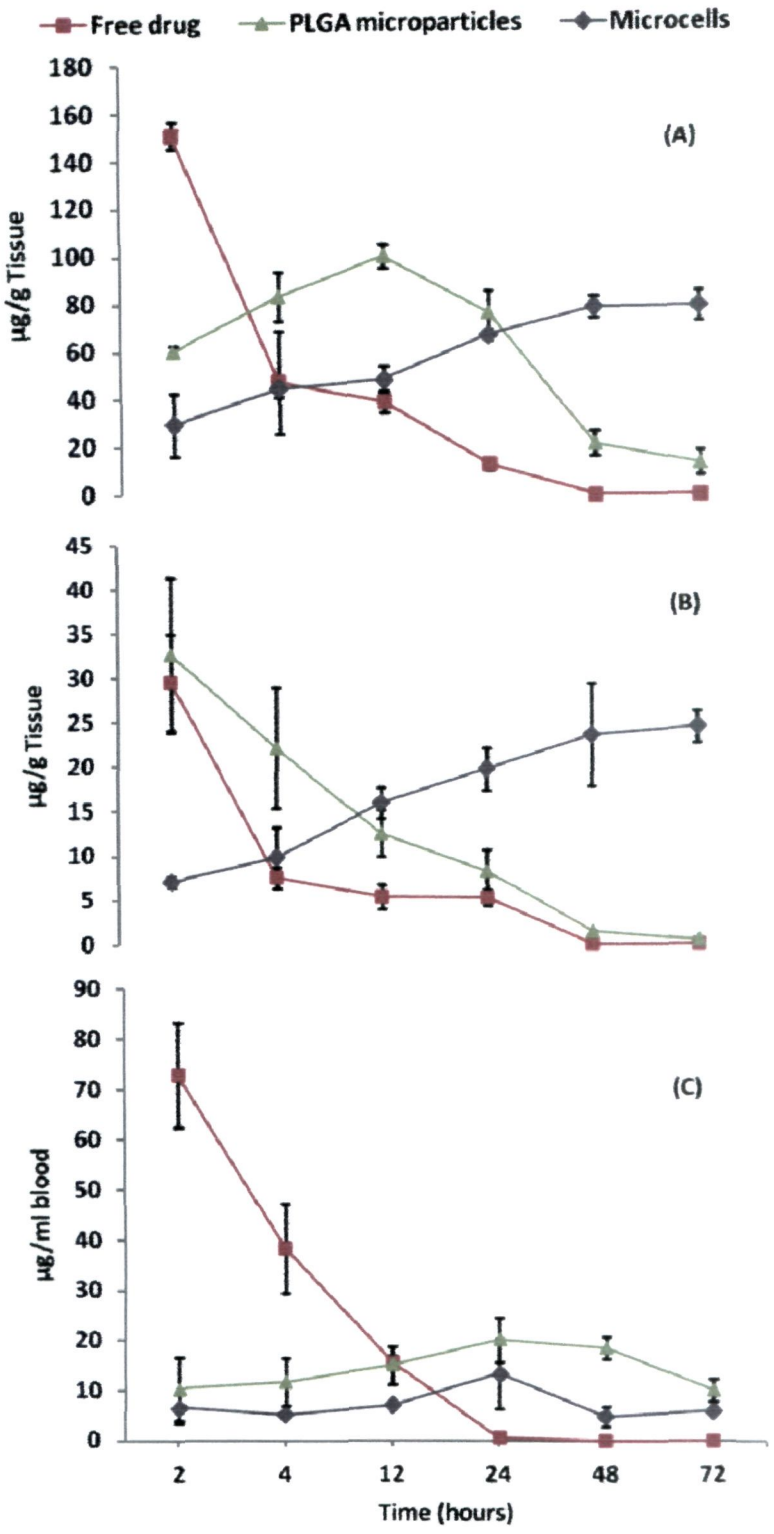


Figure 3.4 Bio-distribution of curcumin from different formulations in various vital organs and blood of treated animals. (A) Liver, (B) Kidney and (C) blood. Curcumin distribution was analysed as described in materials and methods section for a period of 72 hours.

Curcumin loaded PLGA microparticles and microcells mediated liver enzymes suppression

We measured the activity of some important liver enzymes in treated groups. Alkaline phosphatase activity decreased significantly upon treatment with curcumin bearing microcells when compared with free curcumin (microcells *versus* free curcumin; $p<0.01$). Curcumin PLGA microparticles also showed low level of ALP but it was not significant (curcumin PLGA microparticles *versus* free curcumin; $p<0.07$) **(Figure 3.5A)**.

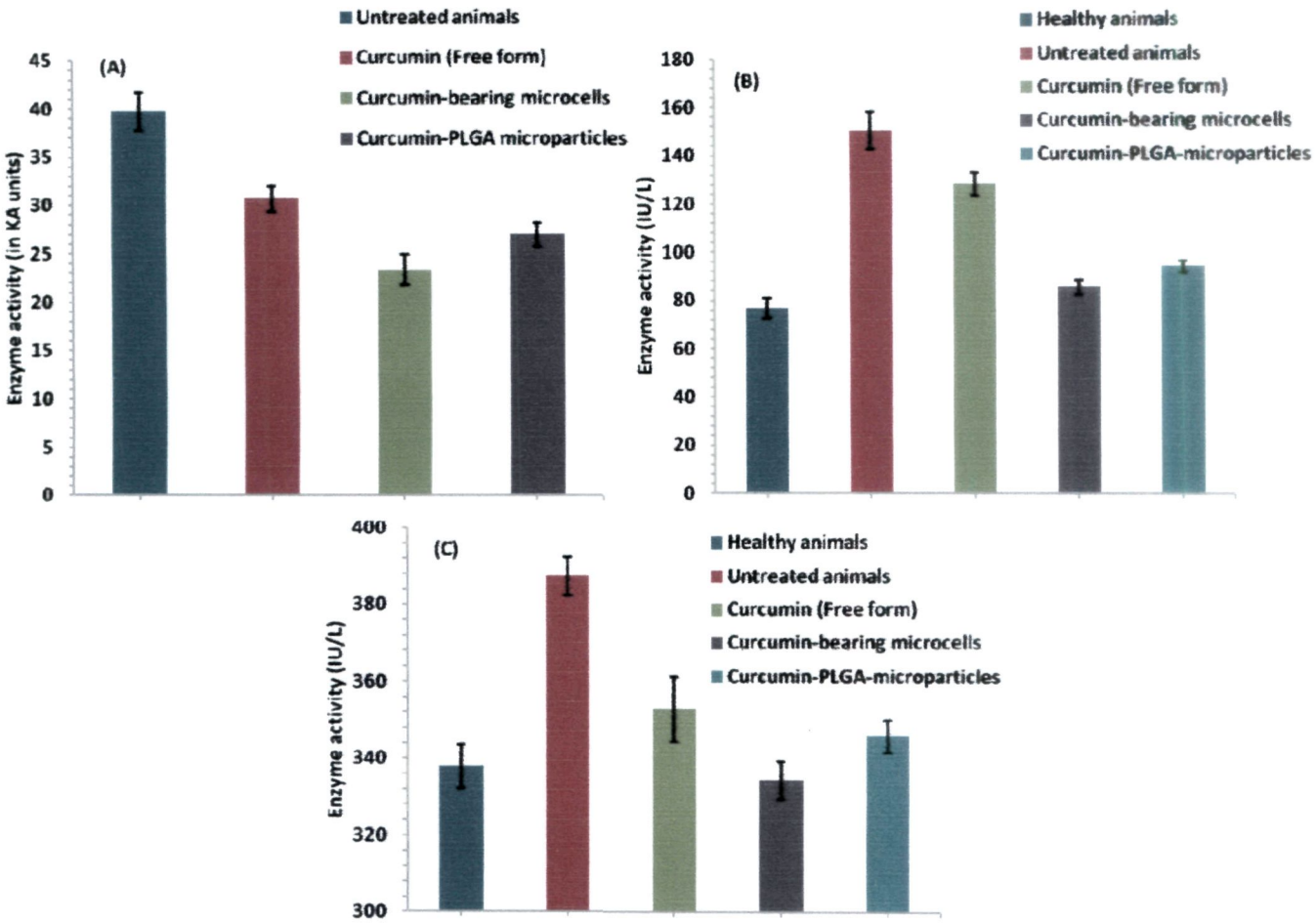


Figure 3.5 Liver enzyme levels in animals treated with various curcumin formulations. Liver functioning enzyme activities were measured as described in materials and methods. Data represented here is mean of three different experiments ± SD. **(A) ALP** (microcells *versus* free curcumin, $p<0.01$; curcumin PLGA microparticles *versus* free curcumin; $p<0.07$) **(B) AST** (microcells *versus* free curcumin, $p<0.05$, curcumin PLGA-microparticle *versus* free curcumin; $p<0.05$) **(C) GGT.**

Further activity of AST was measured in different experimental groups. As shown in **Figure 3.5B** its activity decreased in both curcumin bearing microcells and PLGA-microparticle groups significantly when compared with free curcumin (microcells *versus* free curcumin; $p<0.05$, curcumin PLGA-microparticle *versus* free curcumin; $p<0.05$). We also examined the level of GGT in different treated groups. Although curcumin bearing microcells and PLGA microparticles both showing decreased level of GGT in comparison to free curcumin but the difference was not significant (**Figure 3.5C**).

Curcumin loaded PLGA microparticles and microcells downregulate TNF-α level

Initially Tumor Necrosis Factor was discovered for its anti-tumor activity but now it has been shown to mediate initiation, promotion and tumor metastasis (**Kunnumakkara *et al.* 2008**). Curcumin modulates TNF-α induced signalling and acts as inhibitor of TNF expression (**Aggarwal *et al.* 2006**). In our study we measured TNF-α level in different formulation treated groups. Our results showed that both curcumin bearing microcells and PLGA microparticles, downregulate the TNF-α expression. Although free form of curcumin also downregulated TNF-α expression but it was not as significant as in microcells and microparticles treated groups (curcumin PLGA microparticles *versus* curcumin free from $p<0.05$, microcells *versus* curcumin free from $p<0.05$) (**Figure 3.6**).

Increased survival of curcumin bearing PLGA microparticles and microcells treated animals

We also monitored the survival in different formulation treated groups for a period of 12 weeks. PLGA with molar ratio 50:50, leads to sustained release of entrapped molecules as its degradation requires 1-2 months. Although PLGA microparticles also help in release for longer period but its release pattern was faster when compared to microcells. Sustained release pattern of entrapped curcumin increases its bioavailability, thus, enhances its efficacy. Our results demonstrated that curcumin bearing microcells showed most significant survival rate (80%) followed by curcumin-PLGA microparticles (70%) whereas free form of curcumin could maintain it only 20% survival (microcells *versus* curcumin free form; $p<0.001$, curcumin-PLGA-microparticles *versus* curcumin free form; $p<0.001$) (**Figure 3.7**). No animal survived in untreated, sham microcells and sham-PLGA microparticles group beyond 9th week.

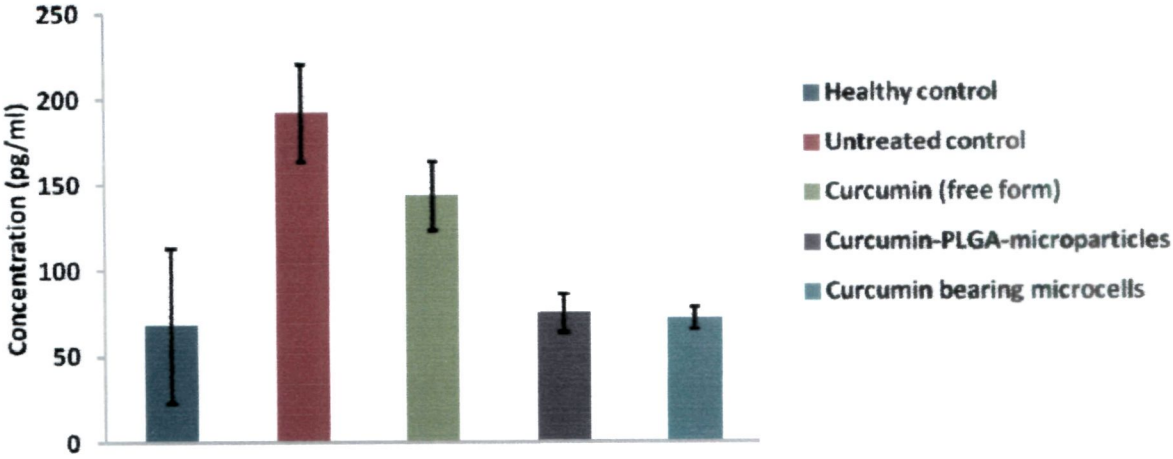


Figure 3.6 TNF-α expression profile of animals treated with different curcumin formulations. TNF-α levels were measured in plasma as described in materials and methods. Data represented here is mean of three different experiments ± SD. (Microcells *versus* free curcumin; $p<0.05$, Curcumin PLGA microparticles *versus* free curcumin; $p<0.05$, Microcells *versus* Curcumin PLGA microparticles; *p value* not significant).

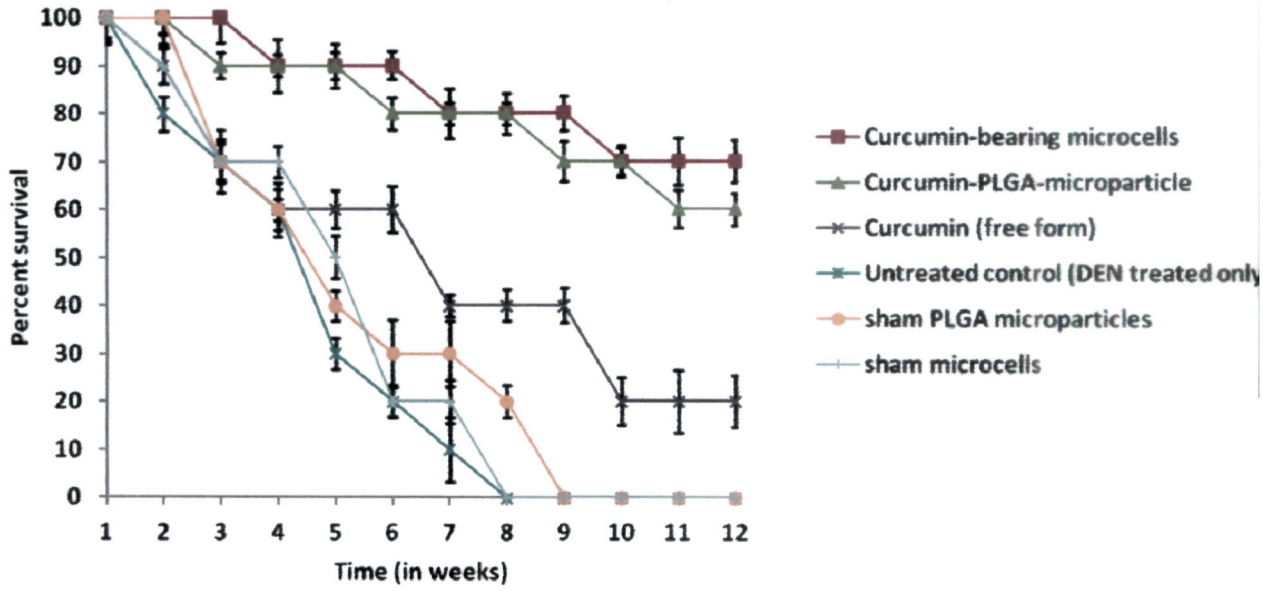


Figure 3.7 Survival graph of different formulations treated experimental groups. Survival was monitored twice a day for a period of 12 weeks. Each group contained 10 animals. Data represented here is mean of three different experiments ± SD. (Microcells *versus* free curcumin; $p<0.005$, Curcumin PLGA microparticles *versus* free curcumin; $p<0.005$, Microcells *versus* Curcumin PLGA microparticles; *p value* not significant).

Histopathological analysis

As compared to healthy control, untreated group reveals impressive inductive effect of carcinogen on liver. Free compound group shows regression but is associated with poorly preserved hepatocellular architecture. Curcumin bearing microparticles group has only mild effect but microcells encapsulated curcumin has moderate therapeutic effect. Sham PLGA-microparticles and sham microcells behaved as untreated group. Thus, if other factors are not taken into account then curcumin bearing microcells show marked recovery, followed by curcumin PLGA microparticles and then free form of the compound (**Figure 3.8**).

Western blot analysis of apoptotic molecules

In the last section of our experiment, we carried out western blot profiling of various apoptotic molecules to ensure the high rate of cell death in curcumin bearing formulation treated groups. As shown in the **Figure 3.9** curcumin bearing microcells and PLGA microparticles had more inductive effect on liver whereas free form of curcumin had very low expression profile. Sham microcells and PLGA microparticles had the same expression as untreated group had (data not shown). Our data is suggestive of liver cancer regression in curcumin bearing microcells and PLGA microparticles treated animals by enhancing the expression of apoptotic molecules.

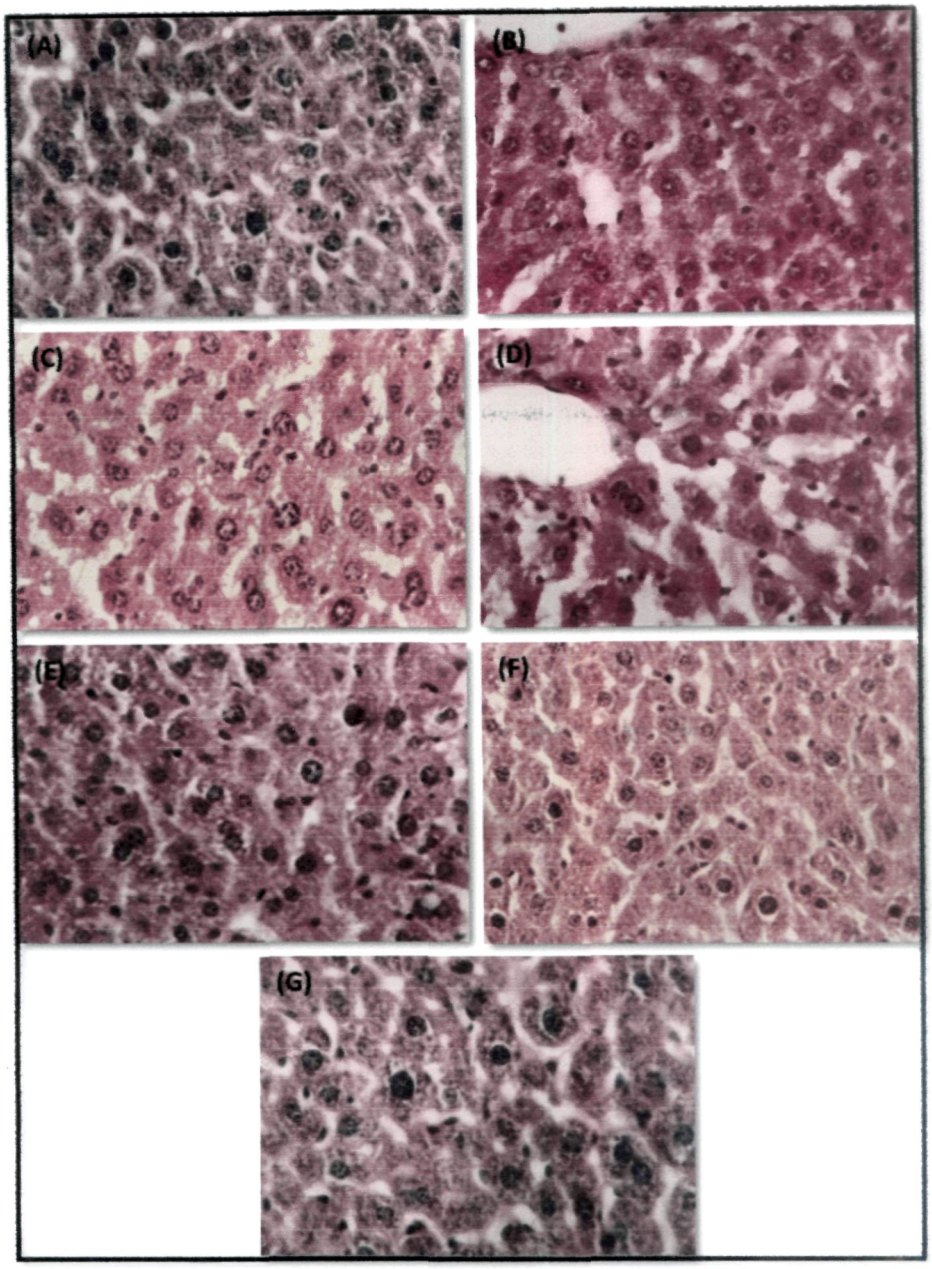


Figure 3.8 Histological analysis of different formulation treated groups. Healthy control: shows normal cords of hepatocytes and sinusoids **(A). Untreated group:** Shows hypercellularity, cellular and nuclear polymorphism and overtly hyperchromatic large nuclei with increase nuclear cytoplasmic ratio **(B).Curcumin bearing microcells:** Hepatic cords and sinusoids appear very similar to health control **(C). Curcumin bearing PLGA microparticles**: Shows increased cellularity, hyperchromatic nuclei, altered hepatocellular contour, distorted sinusoids **(D). Free curcumin:** Shows reduction in nuclear cytoplasmic ratio, poorly maintained hepatocellular contour and dilated sinusoids (E). **Sham microcells and PLGA microparticles:** shows poorly maintained architecture of hepatocytes with cancer growth very similar to untreated control **(F & G)**.

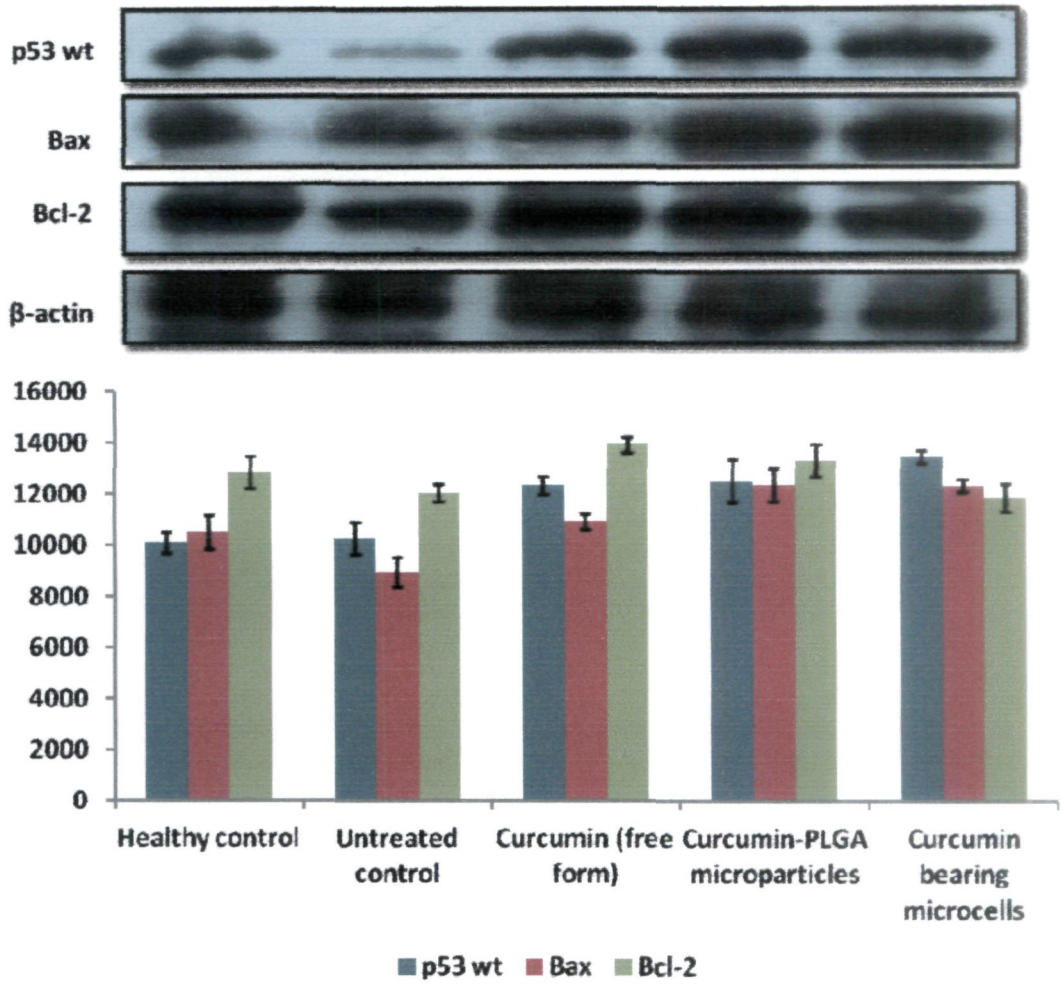


Figure 3.9 Expression profile of apoptotic molecules from various formulation treated groups. Western blot assay of p53 WT, bax and bcl-2 in liver extract from mice treated with various curcumin formulations.

Discussion

Latest advancements in the field of drug delivery have lead to the development of novel biocompatible and biodegradable drug delivery systems capable of targeting cells and tissues, specifically, without affecting adjacent cell types. They can be used for either active or passive targeting. In a quest to develop a nanocarrier for the delivery of hydrophobic curcumin, we have synthesized microcells and PLGA microparticles. Our study ascertains that microcells and curcumin bearing PLGA microparticles besides being safe and free of any toxicity constrains, increase bioavailability and in turn their absorption in tissues. Further, we showed their potential as curcumin carrier in treatment of hepatocellular carcinoma in model animals.

In the past decade, there have been numerous studies done to evaluate potency of curcumin against human cancer cell lines (**Duvoix** *et al.* **2005, Aggarwal** *et al.* **2003, Aggarwal & Shishodia 2006, Choudhuri** *et al.* **2005**). Though curcumin showed very strong activity against the cell lines examined but as a consequence of poor adsorption and fast elimination from body it could not show very promising results *in vivo*. In the present study, we report synthesis of microcells, which were prepared by entrapping the curcumin bearing microparticles in blank PC liposomes. We showed that PLGA microparticles size increased upon encapsulation in PC liposomes (**Figure 3.2A**). Owing to their comparative lesser stability, PLGA microparticles showed faster release than microcells as they lack dual layer of PC liposomes (**Figure 3.2 B**). As shown by release kinetics performed at 37°C, both the delivery systems exhibit time dependent release pattern. Curcumin bearing microcells possessed much sustained release pattern than PLGA microparticles alone. Keeping into consideration that before application of any formulation, it is a pre-requisite to evaluate its toxicity *in vitro* as well as *in vivo*, we performed various studies for any inherent toxicity issues. Curcumin bearing microcells and PLGA microparticles were found to be non-toxic as revealed by *in vitro* RBC lysis test (**Figure 3.3A**). Further, it is also confirmed by *in vivo* LFT and RFT parameters, free curcumin as well as microcells and PLGA microparticles entrapped curcumin did not possess any toxicity at therapeutic dosage (**Figure 3.3B**). Before translating curcumin based formulation of microcells and PLGA microparticles *in vivo*, we also examined bio-distribution of curcumin from various formulations. As shown in results, in free curcumin treated group, curcumin could not be detected in either liver or kidney beyond 24 hours post treatment, although a very low serum concentration was seen in serum in microcells and PLGA microparticles treated groups (**Figure 3.4C**). In PLGA

microparticles treated group curcumin concentration was relatively higher in all the organs examined. Initially its concentration increased but declined after a particular point. Curcumin bearing microcells maintained release pattern in a very much sustained manner that showed increasing curcumin distribution upto 48 hours (**Figure 3.4A & B**). In serum, it exhibited a similar release pattern as PLGA microparticles.

In the subsequent phase of our studies, we induced hepatocellular carcinoma using DEN in model animals. We evaluated liver enzymes (ALP, AST and GGT) and TNF-α expression in treated animals to ascertain the efficacy of our formulations in treating the induced cancer. Curcumin entrapped PLGA microparticles and microcells were found to significantly reduce liver enzymes in comparison to free curcumin (**Figure 3.5**). Most significant reduction was observed in curcumin bearing microcells treated animals which could be attributed to sustained release pattern of curcumin followed by PLGA microparticles. Sham microcells and PLGA microparticles had no effect on liver enzymes and they behaved as untreated group (data not shown). Curcumin also inhibited the activation of NF-κB that in turn reduces the expression of inflammatory cytokines and TNF-α. TNF-α plays an important role in tumor cell survival and its differentiation and metastasis. Our results demonstrated that curcumin encapsulated microcells significantly inhibit the TNF-α expression followed by PLGA microparticles whereas free form of curcumin had very mild effect (**Figure 3.6**). Sham microcells and PLGA microparticles exhibit no effect on TNF-α expression (data not shown).

Survival studies performed for a period of 12 weeks, showed a significant reduction in the mortality of the animals. We analysed that animals treated with curcumin bearing microcells had maximum percent survival (80%) followed by PLGA microparticles group (70%) whereas free form of curcumin showed only 20% survival in the treated animals (**Figure 3.7**). Animals treated with sham microcells and PLGA microparticles did not survive beyond 9th week. Further tissue histology data revealed that curcumin bearing microcells were most effective in maintaining the cellular architecture (**Figure 3.8**). Though PLGA microparticles also showed better effect than free curcumin but it was not comparable to microcells treated group. Sham microcells and PLGA microparticles behaved as untreated group (data not shown).

Mutation in p53 tumor suppressor gene is a significant alteration in apoptosis pathway. Cells with mutated p53 gene result in functional inactivation of p53 and loose the

genomic integrity and escapes from apoptosis (**Soussi** 2000). Bcl-2 family of proteins impart in the apoptosis and share a balance in the cell. Bax, which is a dominant inhibitor of Bcl-2, induces apoptosis by its over-expression and mitochondrial damage. Further, it releases apoptosis mediators and cytochrome C and ultimately leads to cell death (**Cory *et al.* 2003**). With the aim to examine apoptosis, we evaluated expression of p53 WT and bax in the liver tissue extract of various curcumin treated groups. Western blot analysis of apoptotic molecules, p53WT and bax, also revealed that curcumin encapsulated microcells induced the expression of p53WT maximally followed by PLGA microparticles. Free form of curcumin was found to have very mild effect on expression of apoptotic molecules. Bax expression was upregulated in both curcumin entrapped PLGA microparticles as well as microcells treated groups (**Figure** 3.9). Sham PLGA microcells and PLGA microparticles had no effect on expression of either p53WT or bax expression (data not shown).

Our results demonstrate that curcumin's bioavailability increases upon encapsulation into PLGA microparticles and microcells. High drug payload in liver improves the therapeutic index of curcumin thus helping in reduction of HCC. Also, it can be concluded that microcells are sought to be more effective than PLGA microparticles. The difference in the chemotherapeutic effect owe to their release kinetics.

Chapter 4:

siRNA bearing nanocells in treatment of hepatocellular carcinoma

Cancer remains the major cause of death in most of the advanced countries and its incidence increases with aging. Cancer is caused by genetic instability and multiple molecular alterations (**Hanahan & Weinberg 2000, Hahn & Weinberg 2002**). As most of the diagnosis and treatment modalities available are inefficient, it is very difficult to treat cancer.

Chemotherapeutic drugs that are available for the treatment do not differentiate between cancerous and healthy cells robustly, hence, their unsystematic distribution leads to severe toxicity and hampers anti-cancer efficacy. Due to internal genetic diversity and having the ability to mutate rapidly, tumors turn to multi drug resistance, this is a more complicated issue to tackle. Also faster clearance of therapeutics from host body does not allow attainment of desired concentration thus reducing potential of therapy. To overcome those drawbacks, efforts are being made to develop a suitable drug delivery vehicle for instance liposomes, micelles, nanoparticle, and polymer drug conjugates (**Blanco *et al.* 2011**). However these technologies also have certain shortcomings such as *in vivo* drug leakage, premature degradation of delivery vehicle and capacity to encapsulate variety of therapeutics, which make them ineffective for packaging therapeutics (**Vingerhoeds *et al.* 1996**).

Recently discovered RNA interference technology has expanded greatly and emerged as powerful tool to treat cancer (**Fire *et al.* 1998**). The chief advantage of RNAi technology is to target specific disease causing sequences and knock down their expression. As RNAi technology can be used to abolish function of any specific gene it can also be applied to target the genes which play crucial role in cell survival. Cell survival plays a major role in cancer cells as they do have potential to bypass apoptosis and possess self sufficiency in growth factors to promote cell division and differentiation. There are various genes reported that can be targeted to block cell survival (**Kim *et al.* 2007, Xerri *et al.* 1998, Lacasse *et al.* 2005, Peltenburg *et al.* 2000, O'Neil *et al.* 2004**).

COX-2 has effect on many processes that are important in cancer development, such as angiogenesis, apoptosis, inflammation, immunosuppression and invasiveness (**Dannenberg *et al.* 2001**). Overexpression of COX-2 induces vascular growth factor production thus angiogenesis enhancement and apoptosis inhibition by alteration in apoptosis signaling molecules. It had been shown to be upregulated in a variety of human cancers including colon, gastric, esophagus, pancreas and breast cancer, while

undetectable in most normal tissues (**Eberhart** *et al.* 1994, **Subbaramaiah** *et al.* 1996, **Ristimäki** *et al.* 1997, **Hida** *et al.* 1998, **Tucker** *et al.* 1999, **Zimmerman** *et al.* 1999). Furthermore, overexpression of COX-2 was sufficient to cause tumorigenesis in animal models and to render cells resistant to apoptotic stimuli (**McGinty** *et al.* 2000, **Lin** *et al.* 2001, **Cao & Prescott** 2002). Its role in cell survival also has been established (**Oshima** *et al.* 1996, **Sawaoka** *et al.* 1998). Taking these factors into consideration, we chose COX-2 for the treatment of hepatocellular carcinoma in model animals.

In spite of gene silencing using siRNA, a wonderful technology, there are few challenges to be met yet. Innate immune system stimulation (**Marques & Williams** 2005), non specific targeting (**Kleinman** *et al.* 2008), its rapid renal clearance and enzymatic degradation (**John** *et al.* 2007) are the main hurdles to be overcome. The last, but the most important, challenge in siRNA based therapy is the issue of delivery. RNA being anionic and hydrophobic in nature does not enter cells by diffusion. Moreover, it's *in vivo* delivery at disease site is a major problem because naked siRNA is more prone to enzymatic degradation in plasma. Other associated problems like limited penetration across the capillary endothelium and inefficient uptake by tissues further complicate the issue. To overcome these difficulties, it is desirable to develop a suitable and effective delivery system. Although for siRNA delivery into the cytoplasm of cells, many vectors have been developed which are capable of delivering the contents *in vitro* but there is still need of a delivery vehicle that could deliver siRNA *in vivo*. Currently, siRNAs in clinical trials are directly administered to local target sites thus avoiding the complexity of systemic delivery. However, it is essential to introduce siRNA by a systemic route to treat most cancers and other diseases. A desirable delivery system should be biocompatible, biodegradable, and non-immunogenic. Second, the systems should provide optimum delivery of siRNA into target cells or tissues with protection of the active double-stranded siRNA products from attack by serum nucleases. The siRNA delivery should be tissue targeted after its systemic administration, avoiding rapid hepatic or renal clearance. Finally, after delivery into target cells via endocytosis, the system should promote the endosomal release of siRNA into the cytoplasm, allowing the interaction of siRNA with the endogenous RISC.

In a quest to fulfil these requirements, we developed a delivery vehicle from *Bacillus subtilis (B.subtilis)* lipid which mimics as nanocell, and phosphatidyl choline (PC) based liposomes for the delivery of COX-2. *B.subtilis* lipid nanocells (Subtilosomes) and PC liposomes are biocompatible, biodegradable and do not induce any immune response. Also they have been used widely for drug and vaccine delivery application against infectious diseases and cancers as well (**Deeba *et al.* 2005, Maroof *et al.* 2010, Khan *et al.* 2007**). In the present study, we examined the efficacy of subtilosomes and liposomes for the delivery of COX-2 siRNA in treatment of di ethyl nitrosamine (DEN) induced hepatocellular carcinoma (HCC) in model animals.

Materials and methods

All reagents used in the study were of highest purity available. siRNA, was purchased from Santa Cruz Company (USA). Anti-p53 wild type (wt), anti-bax, anti-Cox-2 and anti-β-actin antibodies were purchased from BD Biosciences (San Diego, CA). Liver enzymes were estimated using the kits from Span diagnostics (India). All others reagents were of analytical grade and procured from local suppliers.

B. subtilis lipids isolation, nanocell preparation and siRNA encapsulation

B. subtilis was cultured in nutrient broth (1% peptone, 0.3% beef extract, 0.3% yeast extract and 1% sodium chloride; pH 7.4). The cells were harvested from mid-log phase (18-20 hours). Phospholipids were isolated by the method of Bligh Dyer, as modified by Kumar and Gupta (**Kumar & Gupta 1983**). The nanocells were prepared as described elsewhere (**Ahmad *et al.* 2001**). siRNA was encapsulated using freeze and thaw method.

Encapsulation of siRNA in PC liposomes by dehydration-rehydartion method

siRNA encapsulated PC liposomes were prepared from egg PC (49 μmol) and cholesterol (21 μmol) using published method with some modifications as standardized in our laboratory (**Khan *et al.* 2004**). Briefly, lipids (20 mg) were dissolved in a minimum volume of chloroform:methanol (1:1 v/v). The solvents were evaporated carefully under reduced pressure to form a thin lipid film on the wall of a round bottom flask. Finally, traces of organic solvents were removed by subjecting the flask to high vacuum overnight at 4°C. Subsequently, the dried lipid film was hydrated with 2.0 mL of 150 mM sterile normal saline with intermittent vigorous agitation followed by sonication (30 minutes, 4°C) in a bath type sonicator under nitrogen atmosphere. The sonicated preparation was dialyzed against normal saline for 24 hours at 4°C in the dark, and then centrifuged at 10,000 X g for 30 minutes at 4°C to remove un-dispersed lipid. Now, PB containing siRNA was mixed with PC liposomes. The mixture was frozen and thawed several times and finally lyophilized to get free flowing dry powder. The powder was reconstituted with distilled water ($1/10^{th}$ volume of the original solution). Thus prepared liposomal formulation of siRNA was used for further studies.

Entrapment efficiency of siRNA in liposomes

Entrapment of siRNA in nanoparticles was assessed by the protocol published elsewhere and as standardized in our laboratory. Briefly, an aliquot of prepared liposomes was dissolved in 0.1% Triton X-100 followed by analysis of siRNA content by taking absorbance with UV spectrophotometer (Shimadzu). The percent entrapment efficiency (% EE) was calculated with the following formula.

% EE = (Amount of siRNA entrapped)/(Total amount of siRNA used in the beginning) X 100

Animals

Female Swiss albino mice of weight 20±2 were obtained from the institute's animal house facility. The animals were housed in poly propylene cages on wood powder bedding in an air conditioned ambience. Animals were quarantined on equal light/dark cycles (12/12 hour) and were kept on a pellet diet (Ashirwad, Chandigarh, India) and water *ad libitum*. Animals were examined for their mortality and morbidity prior to commencement of the study and only healthy animals were included in the experiments. The techniques used for administration of various formulations to animals were strictly performed following mandates approved by the animal ethics committee (Committee for the Purpose of Control and Supervision of Experiments on animals, Government of India).

Induction of Liver cancer by di-ethyl nitrosamine (DEN)

Liver cancer in experimental models was induced as described elsewhere and as standardized in our laboratory (**Pitot *et al.* 1978**). Briefly, single dose of DEN (120 mg/kg b.w.) was injected by intraperitoneal route. After incubation of 40 days, animals were given the treatment. Liver cancer induction was ascertained by liver enzyme estimation and tissue histology.

Assessment of anticancer efficacy

Efficacy of siRNA based liposomal formulations was assessed by examining various parameters including liver enzymes, percent survival, liver histology and western blot

profile of apoptotic molecules and Cox-2. siRNA was used at a dose of 100nM/100μl per animal. All formulations of siRNA were given through *i.v* route (intravenous) for a period of 10 days after induction of tumor and then were left for resting period (15 days) to observe the effect of formulations. For our studies, animals were divided into following 7 groups and each experimental group had 10 animals.

Group I Healthy control
Group II Untreated control (DEN treated only)
Group III Sham liposomes
Group IV Sham nanocells
Group V siRNA (free form)
Group VI liposome siRNA
Group VII nanocell siRNA

Assessment of Liver enzymes

It had been shown that liver enzymes get up-regulated in cancerous conditions (**Carr** *et al.* 2010). Hence efficacy of curcumin based nanoparticle formulations was assessed by monitoring the level of different liver enzymes viz; aspartate transaminase (AST) and alanine transaminase (ALT) in treated animals.

Preparation of whole cell fraction

The liver was removed from experimental mice and was placed on ice. Sample was homogenized and the cell fraction was prepared using RIPA buffer.

Western Blotting

The nuclear fraction was analyzed for the presence of various apoptotic molecules using the Western blotting method (**Towbin** *et al.* 1979). Briefly, the protein content of the homogenate was estimated by the routine method using bovine serum albumin as a standard (**Lowry** *et al.* 1951). Proteins (30μg/well) were resolved under denaturing conditions on 10% SDS-PAGE and electro blotted onto nitrocellulose membranes. The blots were blocked overnight with 5% non-fat dry milk and probed with appropriate antibodies at the dilutions recommended by the suppliers. To quantify equal loading, membranes were reported with α-tubulin antibody. Data are presented as the relative

pixel density of each band normalized to a band of α-tubulin. The intensity of the band was quantitated using image analysis software an Image Gel Documentation System.

Apoptosis detection with APO-BRDU labeling

Liver cells were isolated using the protocol described and modified in our laboratory (Klaunig *et al.* 1981). Apoptosis detection from various formulation treated groups was performed using Brdu staining kit (APO-BRDU kit, BD biosciences). Briefly, 1 X 10^6 cells/mL were suspended using 1% paraformaldehyde (dissolved in PBS pH 7.4). Suspended cells were placed on ice for 30-60 minutes. After fixation, cells were centrifuged at 500 x g for 5 minutes and supernatant was discarded. Cells were washed with PBS thrice and resuspended in 70% (v/v) ice cold ethanol. Cells were incubated on ice for 30 minutes. Cells were centrifuged at 300 x g for 5 minutes and ethanol was aspirated. Now cells were washed using wash buffer thrice and cell pellet was incubated with 50 μl DNA labeling solution at 37°C for 60 minutes. At the end of incubation period, 1.0 mL of rinse buffer was added and tubes were centrifuged at 300 x g for 5 minutes. This was repeated three times and finally cell pellet was incubated with 0.1 mL of FITC labeled anti-Brdu antibody in the dark for 30 minutes at room temperature. Now cells were analyzed PI/RNase staining buffer by flow cytometry.

Histological study

To examine effect of siRNA entrapped nanoparticles on the development of tumor, three mice from each group were sacrificed by cervical dislocation and fixed in 10% formaldehyde solution. Tumor tissues were then excised and sections were prepared by using conventional paraffin sections and hemotoxylin-eosin staining.

Statistical analysis

One way ANOVA was used for comparing the mean values of tumor volume between various groups after ascertaining the homogeneity of variance between treatments. Post hoc analysis for comparing the two groups was done using the Least Statistical Difference (LSD) technique. The Kaplan-Meier analysis was used to estimate survival of tumor free animals and differences were analyzed by log-rank test.

Results

COX-2 encapsulated liposomes mediated liver enzymes depletion

It has been shown that entrapment of siRNA in a nanocarrier not only circumvents its hydrophobicity but also delivers the contents in the cytosol of the cell and renders protection from premature degradation (Kim *et al.* 2008). Nanocells constructed with *B. subtilis* total lipid had entrapment 27±4.2% and in case of PC liposomes it was 23±2only. In our studies we examined COX-2 siRNA delivery for the treatment of HCC. COX-2 has been shown to mediate the process of cancer proliferation. As a parameter, we examined activity of ALT and AST in the serum of animals treated with various siRNA based formulations. Our data showed that nanocells mediated delivery of COX-2 inhibits cell survival significantly and in turn downregulates the enzymes activity. Liposomes also helped in cancer regression but its activity was not comparable to nanocells. Naked siRNA had very little effect on the enzyme reduction (**Figure 4.1**).

COX-2 mediated apoptosis induction in various siRNA treated groups

In another set of experiment, to evaluate COX-2 mediated apoptosis, we isolated the liver cells and stained for Brdu and observed with flow cytometry. Our results established that nanocells were most effective in delivering their content at targeted site and had maximum rate of apoptosis (18.53%) followed by liposomes (10.48%). Naked siRNA had only mild effect on apoptosis induction and it was only 7.53% **Figure 4.2(I) and (II)**. Sham nanocells and liposomes had no effect on apoptosis. So, our results further demonstrated the superiority of nanocells over liposomes in delivering COX-2 siRNA.

Western blotting of apoptotic factors

We also performed Western blotting of COX-2 and apoptotic molecules (p53 wt and Bax). As shown in figure 4.3, nanocells showed very little expression of COX-2 followed by liposomes whereas naked siRNA had expression similar to sham nanocells, sham liposomes and untreated control groups. So our data clearly indicates the safe delivery of COX-2. COX-2 mediated cell arrest was further confirmed by expression pattern of p53 wt and Bax. p53 wt expression was induced in nanocells COX-2 siRNA significantly. Although liposomal siRNA group also had slightly high expression of p53 wt but not comparable to nanocells entrapped siRNA (**Figure 4.3**). Naked siRNA behaved as vehicle controls and had similar expression profile (**Figure 4.3**).

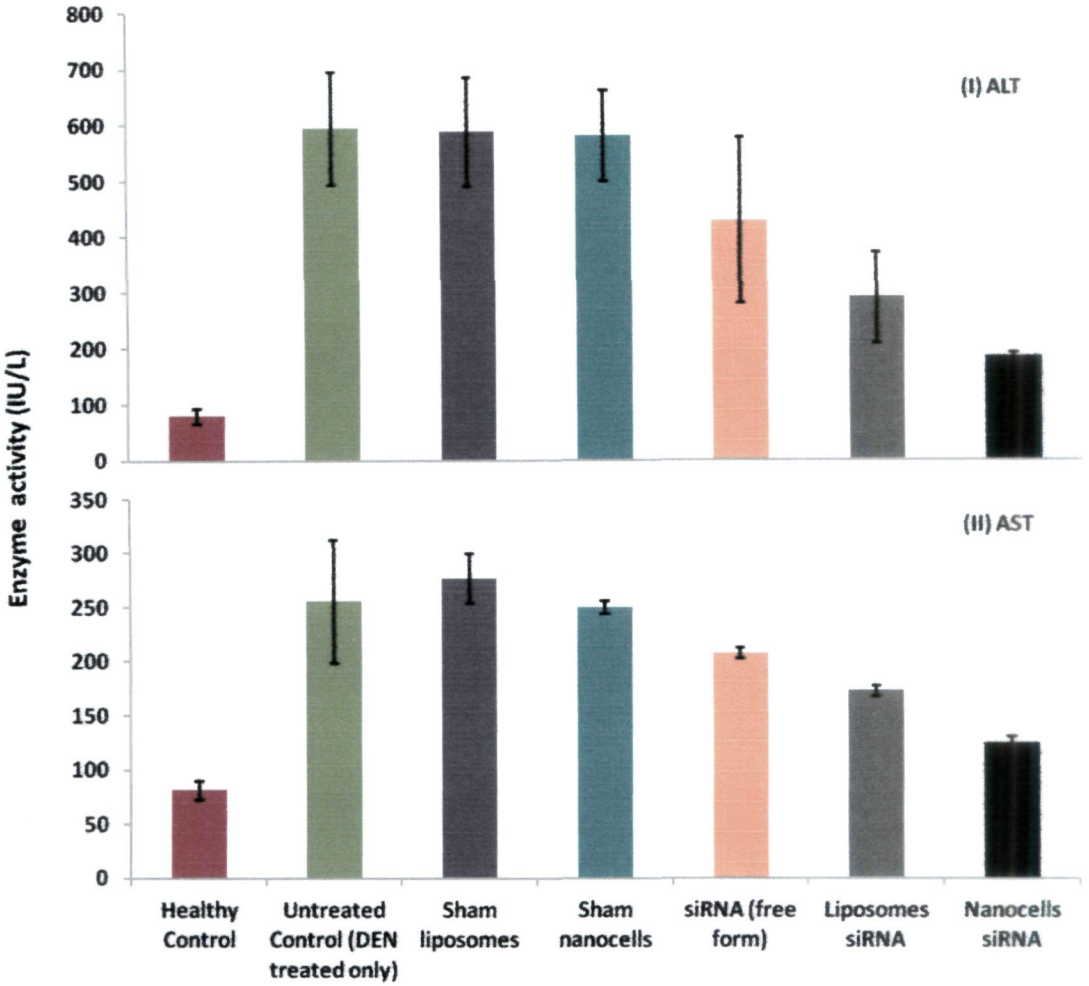


Figure 4.1 Liver enzymes levels in animals treated with different siRNA bearing formulations. Enzyme activities were measured as described in materials and methods. Data represented here is mean of three different experiments ± SD. **(I) ALT** (Nanocells siRNA *versus* free siRNA; $p<0.05$, liposomes siRNA *versus* free siRNA; *p value* not significant, nanocells siRNA *versus* PC liposomes siRNA; $p<0.05$), **(II) AST** (nanocells siRNA *versus* free siRNA; $p<0.005$, liposomes siRNA *versus* free siRNA; $p<0.05$, nanocells siRNA *versus* liposomes siRNA; $p<0.005$).

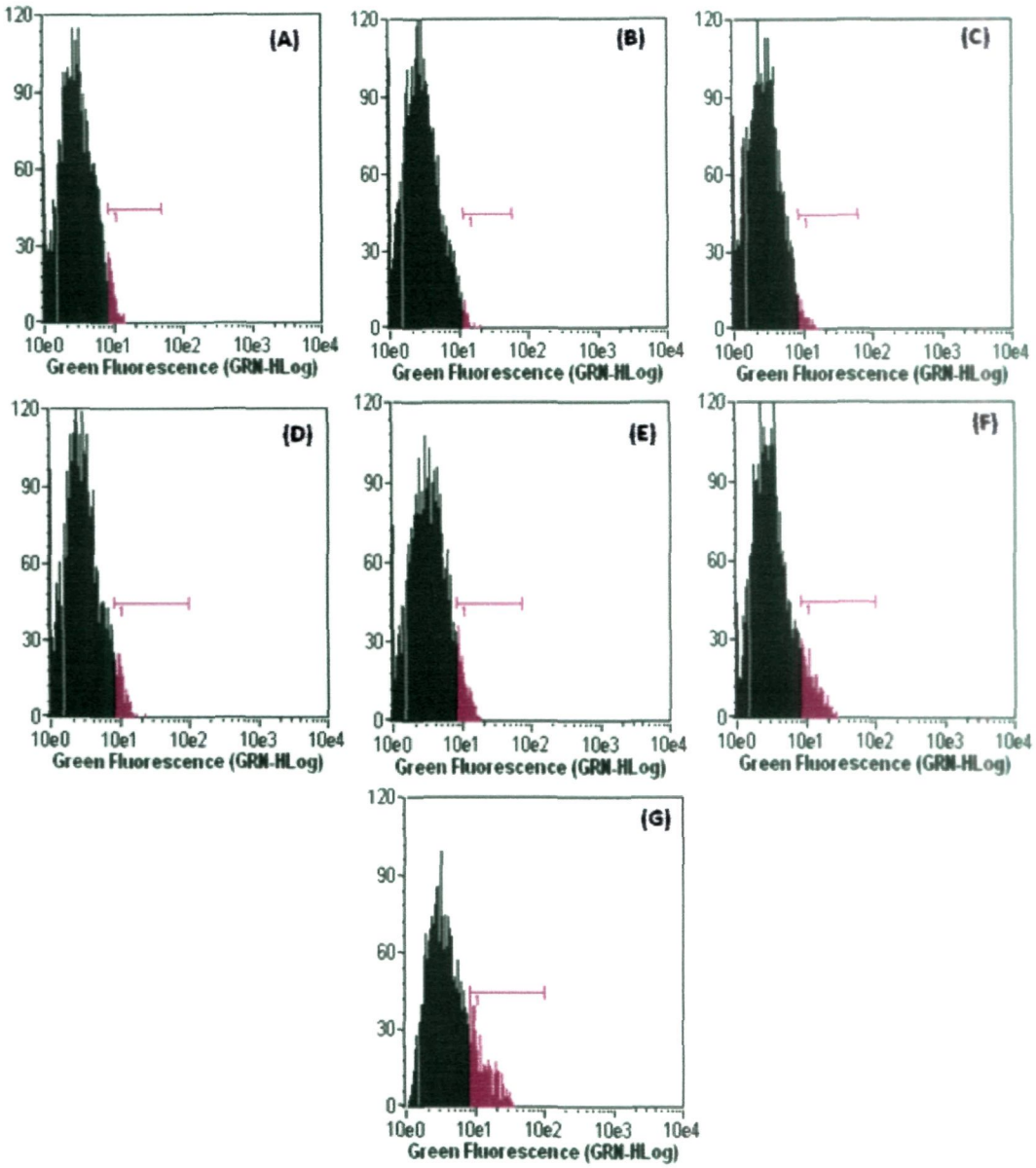


Figure 4.2 Apoptosis in liver cells analyzed by Brdu staining. Staining was performed using Brdu staining kit (BD biosciences) as described in materials and methods section. **Figure 4.2(I)** shows stained cell population from various formulation treated groups.

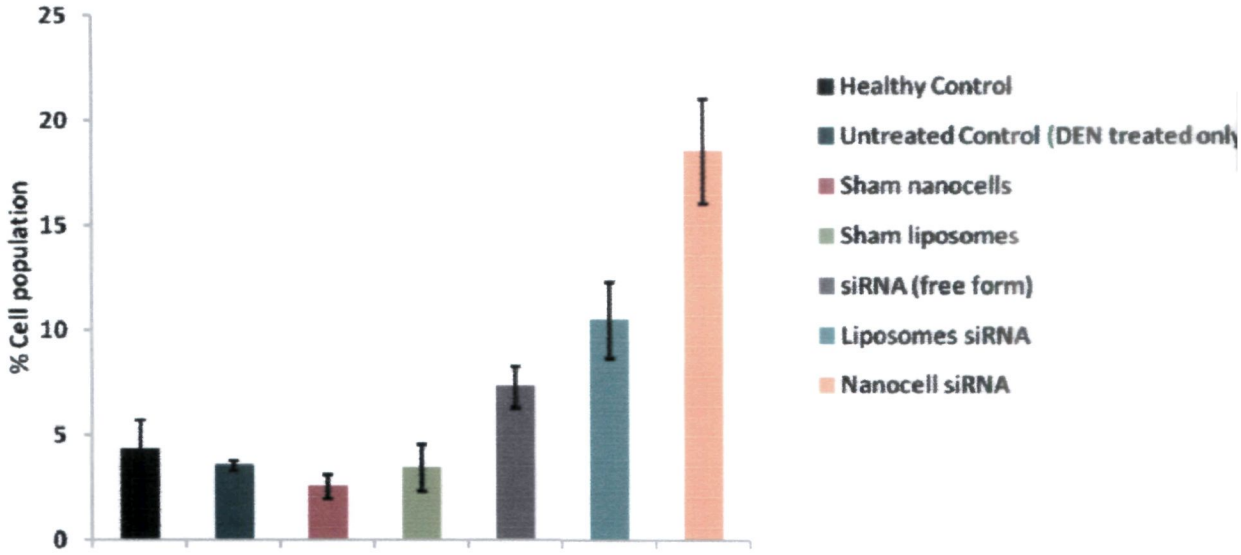


Figure 4.2(II) depicts total percent cell population observed in different formulation treated groups.

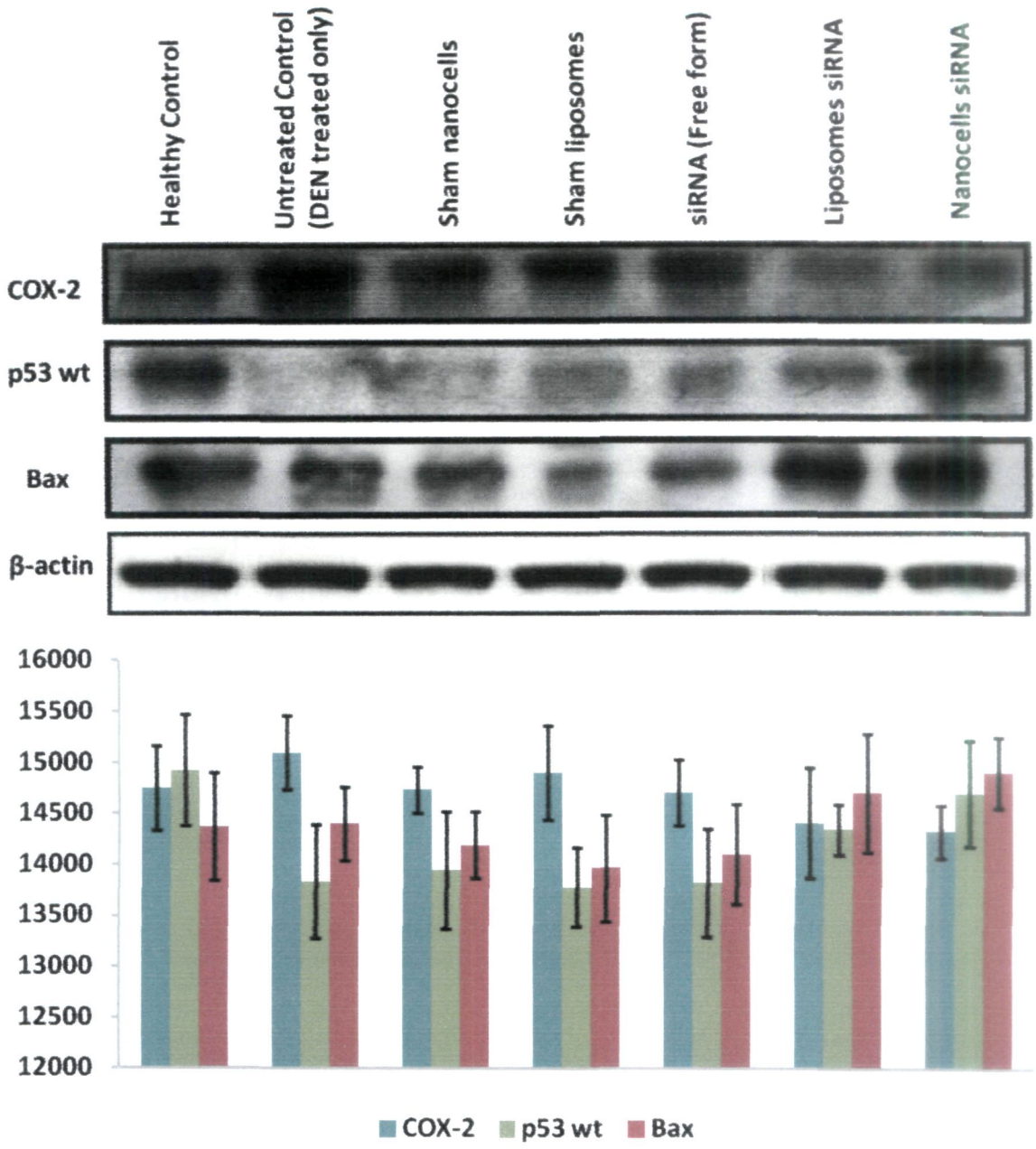


Figure 4.3 Expression profile of COX-2 and various apoptotic molecules from various formulation treated groups. Western blot assay of COX-2, p53 wt and Bax in liver extract from mice treated with various COX-2 siRNA bearing formulations.

Histology of liver tissue treated with siRNA based formulations

Further histology of the liver tissue samples from various treated groups ascertained that nanocells were capable of delivering the COX-2 siRNA in liver tissues. Though free form of siRNA also had little effect on regression but not as significant as in case of nanocells and liposome based formulations (**Figure 4.4**). Sham nanocells and sham liposomes behaved as untreated control and showed very poor cellular architecture of liver tissue in treated animals (data not shown). Nanocells siRNA and liposomes siRNA appear to have definite therapeutic role in prevention/treatment of induced liver cancer.

Survival study

HCC bearing animals treated with various formulations were monitored for their survival for a period of 12 weeks. In accordance of previous results nanocells entrapped COX-2 siRNA had maximum survival rate (80%) followed by liposomes encapsulated siRNA (50%). Naked siRNA as more prone to enzymatic degradation; had 20% survival rate only (**Figure 4.5**). Sham nanocells and liposomes treated animals did not survive beyond 9^{th} week post treatment. Our results established that siRNA encapsulation of COX-2 not only provide it serum stability but also delivers the content at targeted site and helps in tumor regression, in turn increasing the survival rate of treated animals.

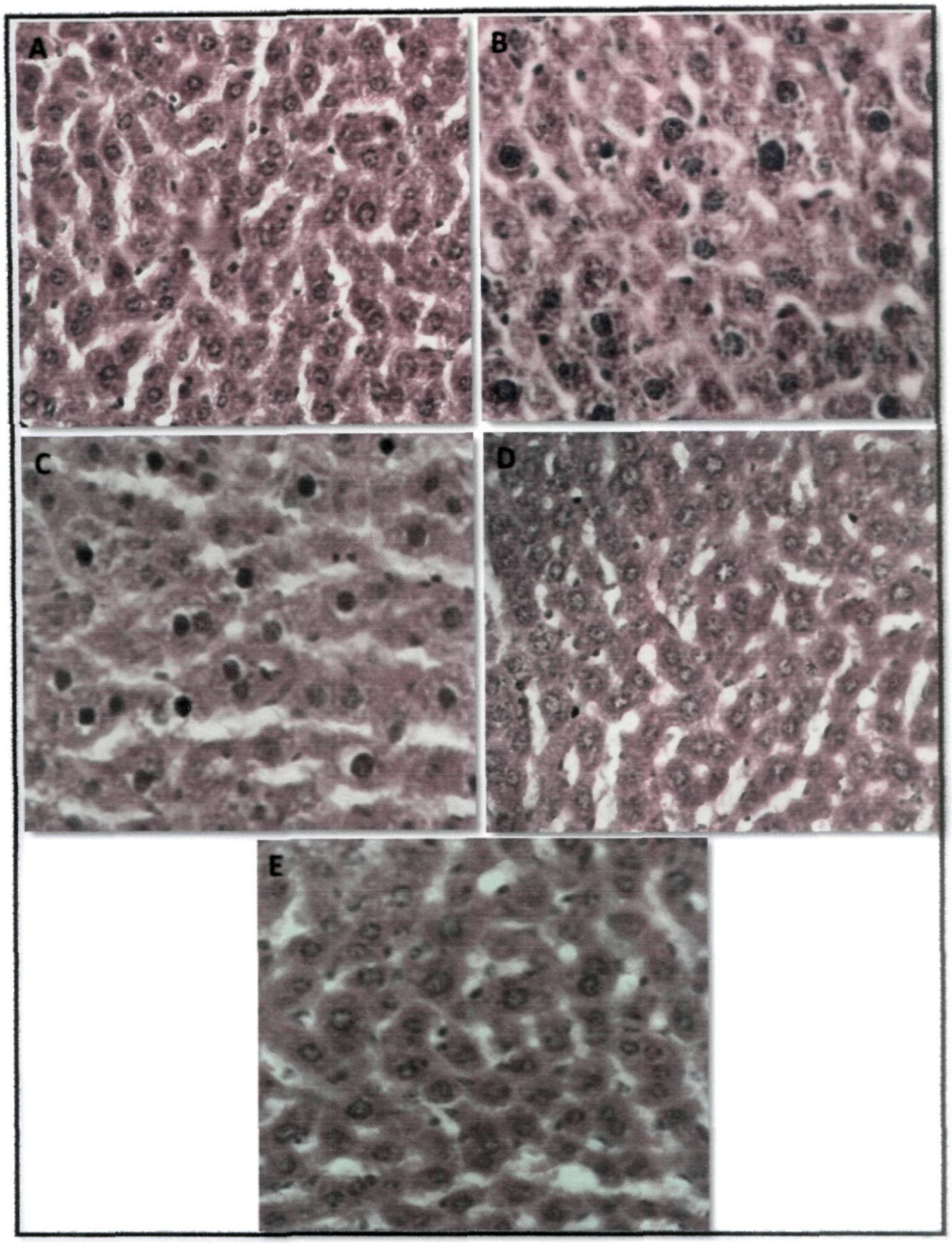


Figure 4.4 Photomicrographs of mice liver treated with various siRNA based formulations. (A) healthy control shows normal hepatocytes, sinusoids, and hepatic cords, **(B) untreated control** shows hypercellularity, cellular and nuclear polymorphism and overtly hyperchromatic large nuclei with increase in the nuclear cytoplsmic ratio; distortion of hepatic cords and compression of sinusoids, **free siRNA (C)** shows only mild suppression but still many hepatocytes very akin to untreated control are visible. **Liposomes siRNA (D)** appears very akin to healthy tissues. Hepatocytes, their nuclei, cytoplasm, hepatic cords, and sinusoids appear normal in **nanocell siRNA (E)** treated group.

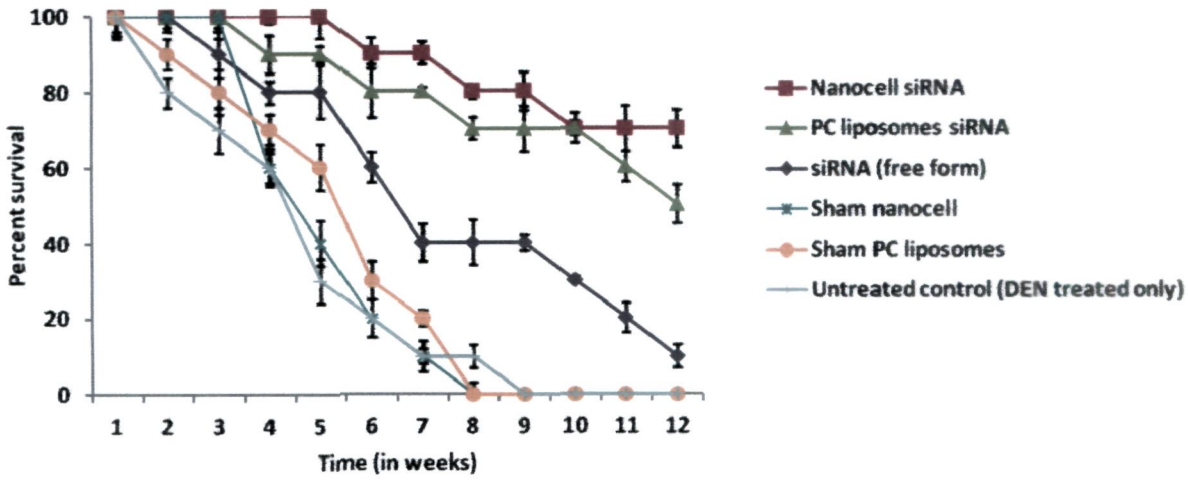


Figure 4.5 Survival graph of different COX-2 siRNA encapsulated formulations treated experimental groups. Survival was monitored twice a day for a period of 12 weeks. Each group had 10 animals. Data represented here is mean of three different experiments ± SD. (Nanocells siRNA *versus* free siRNA; $p<0.005$, liposomes siRNA *versus* free siRNA; $p<0.05$, nanocells siRNA *versus* liposomes siRNA; *p value* not significant).

Discussion

Being specific and potent in activity, RNA interference is emerging as new generation wonder therapeutic. As siRNA inhibits at translation stage not at transcription, they do not interact with chromosomal DNA. This phenomenon reduces the risk that is posed by DNA based gene therapy. Traditional drugs or chemically synthesized drugs only targets specific cell receptors, enzymes, ion channels or ligands. In biological molecule based therapy, only a part of cell surface receptors, tissue or blood component are targeted while siRNA based therapy has advantage of targeting mRNA of interest regardless of protein location. Another tag to add to this technology is that only few siRNA molecules are enough to halt expression of particular gene (**de Fougerolles** *et al.* 2007). But in spite of having these advantages siRNA could not be translated in clinical settings successfully and possess certain issues to be surmounted. Most of the siRNA which are undergoing clinical trials are injected at the diseased tissue or in close proximity. Among various issues, major is to deliver them specifically and another is to provide them stability. Many techniques have been developed like chemical modification, lipid and polymer mediated delivery and polymer conjugates. In one such approach, we developed nanocell from *B. subtilis* lipid and PC liposomes to encapsulate COX-2 for the treatment of DEN induced liver carcinogenesis.

Minicells from bacterial origin were first observed and reported by Adler *et al* in 1967 (**Adler** *et al.* 1967) and later it was shown that minicells derived from bacterial origin have capacity to encapsulate variety of chemotherapeutics (**MacDiarmid** *et al.* 2006). Since minicells are of bacterial origin, it is very much possible that they might induce immune response as bacterial products are known to activate potent inflammatory responses activated by Toll-like receptors (**Shizuo & Takeda 2004**). To avoid inclusion of lipopolysaccharides, we isolated lipid components only and further used them for the synthesis of nanocells.

COX-2 is an important enzyme of prostaglandin synthesis and gets upregulated in various cancer types. This enzyme imparts in carcinogenesis and angiogenesis. Certain inhibitors have been developed to treat COX-2 mediated carcinogenesis (**Kawamori** *et al.* 1998, **Oshima** *et al.* 2001, **Rioux** *et al.* 1998, **Shiotani** *et al.* 1998, **Stolina** *et al.* 2001). But siRNA technology more specificifically provides better alternative to target COX-2. Liposome based delivery of various siRNAs has been reported to be safe and provides serum stability (**Felgner** *et al.* 1989, **Kim** *et al.* 2006). In the present study, we

encapsulated COX-2 siRNA in *B. subtilis* lipid nanocells and PC liposomes, and their efficacy was evaluated against liver carcinogenesis. We measured liver enzymes activity as a parameter to check the liver carcinogenesis and found that nanocells deliver COX-2 much efficiently and helped in liver enzyme (ALT and AST) regression in treated animals (**Figure 4.1**). Being more stable nanocells showed more enzyme reduction than PC liposomes. siRNA in free form is more prone to degradation by serum endonucleases and hence possess very negligible activity. We also estimated apoptosis rate in isolated liver cells of various treated groups. Our results (**Figure 4.2 I**) showed that nanocells were most successful in bringing about apoptosis in liver cancer cells followed by liposomes. The probable explanation for varying activity is their stability and capacity to deliver their content at targeted site. Again naked siRNA delivery did not have much effect on apoptosis induction. Sham liposomes had no effect on apoptosis induction. Percent apoptosis rate was 18.53, 10.43 and 7.52 in nanocells encapsulated COX-2 siRNA, PC liposomes siRNA and free form of siRNA treated groups respectively (**Figure 4.2 II**).

Apart from percent apoptosis measurement, we also elucidated the signalling pathway for cell cycle arrest. Apoptosis and cell cycle regulation are two interlinked processes. Abnormal cell differentiation leads to malignancy development and other abnormalities. Cell differentiation and apoptosis is maintained at molecular level. Every healthy cell maintains equilibrium of those molecules for cell differentiation, survival and apoptosis. p53, being a tumor suppressor gene induces apoptosis in response to DNA damage and saves cell from undergoing carcinogenesis. Bax is another molecule of its downstream signalling and is p53 target for apoptosis induction. So with the aim to analyze this p53 mediated machinery of apoptosis, we performed Western blotting of p53 wt and Bax. We observed that p53 wt expression was maximum in nanocells siRNA treated animals followed by liposomes siRNA. Naked siRNA delivery had very mild effect on its upregulation (**Figure 4.3**). Same expression pattern was observed for Bax too. From those results we can interpret that liposome mediated COX-2 delivery successfully induces apoptosis in liver cancer cells. Further, to validate these results we performed histology of liver tissues of various treated groups and monitored their survival. Histological staining also showed that nanocells mediated COX-2 delivery helped in cellular architecture maintenance. Though liposomes siRNA treated animals also showed better results but not comparable to nanocell siRNA. Free form of siRNA had very wage

effect on carcinogenesis. Sham subtilosomes and liposomes treated animals behaved as untreated control (**Figure 4.4**). In accordance to our previous findings, we observed that nanocells COX-2 siRNA treated animals had 80% survival rate whereas liposomes entrapped siRNA and free siRNA had only 50 and 20% survival rate (**Figure 4.5**).

On the basis of present findings, we can conclude that nanocells being more stable in nature deliver COX-2 siRNA much efficiently than liposomes, helping in cancer regression and increasing survival in treated animals by bringing about apoptosis. We can conclude that *B. subtilis* nanocells mediated siRNA delivery provides serum stability and delivers large numbers of siRNA molecules and thus bears superiority over free delivery of siRNA.

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ORIGINAL RESEARCH

Anticancer efficacy of perillyl alcohol-bearing PLGA microparticles

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Abstract: In the present study, a novel poly-lactic glycolic acid (PLGA)-based microparticle formulation of perillyl alcohol (POH) was prepared and characterized. Further, its efficacy was evaluated against di-methyl benzo anthracene-induced skin papilloma in Swiss albino mice. The characterization studies showed that POH-bearing PLGA microparticles were of the size 768 ± 215 nm with a ζ-potential value of −7.56 ± 0.88 mV. The entrapment efficiency of the active drug in particles was 42.4% ± 3.5%. POH-bearing PLGA microparticles were stable and released entrapped drug gradually over an extended time period. The in vitro efficacy of POH-bearing PLGA microparticles was evaluated by examining their differential cytotoxicity and assessing their ability to inhibit epidermoid carcinoma cell line (A253). The POH-based microparticles when administered to tumor-bearing animals caused greater tumor regression and increased survival rate (~80%) as compared with the group receiving free form of POH (survival rate 40%). The superiority of POH-PLGA microparticles over free form of POH was further evident from their ability to modulate apoptosis-regulating factors.

Keywords: poly-lactic glycolic acid, epidermoid cancer cells, skin papilloma, anticancer efficacy

Introduction

Cytotoxicity and other related side effects are the most serious problems associated with the currently available anticancer drugs. Other limitations include widespread systemic distribution and rapid elimination of the administered anticancer drugs from the host body. A worldwide search therefore continues for anticancer drugs that are more potent, less toxic, and manifest minimum untoward effects to the host. Several plant derived compounds have been reported to possess strong anticancer properties and have been shown to delay, inhibit, or reverse cancerous growth in an effective manner. For example, perillyl alcohol (POH), a plant-based compound, has been reported to possess strong anti-cytotoxic properties against several types of cancer including those of breast, pancreatic, and liver.[1–3] POH is a monoterpene and constituent of essential oils from a number of plants; namely, perilla (*Perilla frutescens*), lavendin, peppermint, ginger grass, savin, caraway, and celery seeds.[4]

Before translating the suitability of a novel compound like POH as a potential anticancer agent in the clinical setting, it is desirable to address some of the associated issues like that of its solubility, palatability, and sustained/controlled release in systemic circulation. This requires designing of a suitable drug-delivery system that can release the drug gradually over a long period of time and, in turn, facilitate its uptake by cancer cells and thereby helps in increasing the efficacy of the entrapped drug. Polymeric

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